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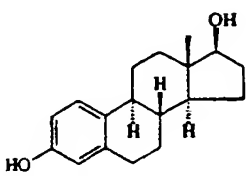

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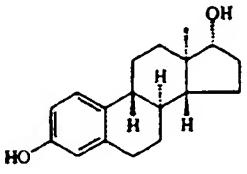


Ent-estradiol

(57) Abstract: The present invention in various embodiments provides methods of cytoprotection and treatment of disease that include providing an enantiomer of an estrogen compound to a population of cells in a subject with a cytodegenerative condition to protect those cells from further damage. Examples of cytodegenerative conditions include stroke and neurodegenerative diseases.

METHODS OF CYTOPROTECTION USING AN ENANTIOMER OF ESTROGEN OF ISCHEMIC DAMAGE

Technical Field

The present invention relates to the protection of cells that would otherwise die as a result of stroke or an ischemic event.

Background

Ischemia is an acute condition associated with an inadequate flow of oxygenated blood to a part of the body, caused by the constriction or blockage of the blood vessels supplying it. Ischemia occurs any time that blood flow to a tissue is reduced below a critical level. This reduction in blood flow can result from: (i) the blockage of a vessel by an embolus (blood clot); (ii) the blockage of a vessel due to atherosclerosis; (iii) the breakage of a blood vessel (a bleeding stroke); (iv) the blockage of a blood vessel due to vasoconstriction such as occurs during vasospasms and possibly, during transient ischemic attacks (TIA) and following subarachnoid hemorrhage. Conditions in which ischemia occurs further include (i) myocardial infarction; (ii) trauma; and (iii) during cardiac and thoracic surgery and neurosurgery (blood flow needs to be reduced or stopped to achieve the aims of surgery). During myocardial infarct, stoppage of the heart or damage occurs which reduces the flow of blood to organs, and ischemia results. Cardiac tissue itself is also subjected to ischemic damage. During various surgeries, reduction of blood flow, clots or air bubbles generated can lead to significant ischemic damage.

When an ischemic event occurs, there is a gradation of injury that arises from the ischemic site. The cells at the site of blood flow restriction, undergo necrosis and form the core of a lesion. A penumbra is formed around the core where the injury is not immediately fatal but progresses slowly toward cell death. This progression to cell death may be reversed upon reestablishment of blood flow within a short time of the ischemic event.

Focal ischemia encompasses cerebrovascular disease (stroke), subarachnoid hemorrhage (SAH) and trauma. Stroke is the third leading cause of morbidity in the United States, with over 500,000 cases per year, including 150,000 deaths annually. Post-stroke sequelae are mortality and debilitating chronic neurological complications which result from neuronal damage for which prevention or treatment are not currently available.

Following a stroke, the core area shows signs of cell death, but cells in the penumbra remain alive for a period of time although malfunctioning and will, in several days, resemble the necrotic core. The neurons in the penumbra seem to malfunction in a graded manner with respect to regional blood flow. As the blood flow is depleted, neurons fall electrically silent, their ionic gradients decay, the cells depolarize and then they die. Endothelial cells of the brain capillaries undergo swelling and the luminal diameter of the capillaries decrease. Associated with these events, the blood brain barrier appears to be disrupted, and an inflammatory response follows which further interrupts blood flow and the access of cells to oxygen.

The effects of a stroke on neurons result from the depletion of energy sources associated with oxygen deprivation which in turn disrupts the critically important ion pumps responsible for electrical signaling and neurotransmitter release. The failure of the ATP-dependant ion specific pumps to maintain ion gradients through active transport of sodium, chlorine, hydrogen, and calcium ions out of the cell and potassium ions into the cell results in a series of adverse biochemical events. For example, increase in intracellular calcium ion levels results in: (i) the production of free radicals that extensively damage lipids and proteins; (ii) the disruption of calcium sensitive receptors such as the N-methyl D-aspartate (NMDA) and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) synaptic glutamate receptors; (iii) the swelling of cells with water as a result of abnormal accumulation of ions; and (iv) the decrease in intracellular pH. The alteration in metabolism within the cell further results in the accumulation of ions in the cells as energy sources are depleted. For example, anaerobic glycolysis that forms lactic acid, replaces the normal aerobic glycolysis pathways in the mitochondria. This results in acidosis that results in further accumulation of calcium ions in the cell.

Despite the frequency of occurrence of ischemia (including stroke) and despite the serious nature of the outcome for the patient, treatments for these conditions have proven to be elusive. There are two basic approaches that have been undertaken to rescue degenerating cells in the penumbra. The first and most effective approach to date has been the identification of blood clot dissolvers that bring about rapid removal of the vascular blockage that restricts blood flow to the cells. Recombinant tissue plasminogen activator (TPA) has been approved by the Federal Drug Administration for use in dissolving clots that cause ischemia in thrombotic stroke. Nevertheless, adverse side effects are associated with the use of TPA. For example, a consequence of the breakdown of blood clots by TPA treatment is cerebral hemorrhaging that results from blood vessel damage caused by the ischemia. A second basic approach to treating degenerating cells deprived of oxygen is to protect the cells from damage that accumulates from the associated energy deficit. To this end, glutamate antagonists and calcium channel antagonists have been most thoroughly investigated. None of these have proven to be substantially efficacious but they are still in early clinical development. The pathophysiology and treatment of focal cerebral ischemia has been reviewed by B.K. Seisjo, J. Neurosurgery, 1992, vol. 77, p. 169-184 and 337-354.

In addition to the targets of drug development described by Seisjo (1992), epidemiological studies have shown that women undergoing hormone replacement therapy with estrogen and progesterone experienced a reduction in the incidence and severity of heart disease. This correlation was further investigated for stroke with mixed results. A 10-year epidemiological study on 48,000 women reported by Stampfer et al. (New England Journal of Medicine, 1991, vol. 325, p. 756) concluded that there was a correlation between use of estrogen and decrease in incidence of coronary heart disease, but no decrease in the incidence of stroke was observed. In contrast, a report by Wren (The Medical Journal of Australia, 1992, vol. 157, p. 204) who reviewed 100 articles directed to the question as to whether estrogens reduce the risk of atherosclerosis and myocardial infarction, concluded that estrogens in hormone replacement therapies significantly reduce the incidence of myocardial infarction and stroke and may accomplish this at the site of the blood vessel wall. This conclusion was further supported by Falkeborn et al. Arch Intern. Med., 1993, vol. 153, p. 1201. The above correlation between estrogen replacement therapy and reduced incidence of stroke relies

on epidemiological data only. No biochemical data were analyzed to interpret or support these conclusions, nor is there any information as to reduction in ischemic lesion or morbidity with hormone use. Furthermore, these studies were restricted to the patients receiving long-term hormone replacement treatment. No studies were performed on patients who might be administered estrogen therapeutically shortly before, during, or after a stroke for the first time. Furthermore, the studies were limited to estrogens utilized in estrogen replacement therapy. No studies were performed on any non-sex related estrogens that might be used in treating males or females.

Studies have been conducted on the neuroprotective effects of steroids in which glucocorticosteroid for example was found to have a positive effect in reducing spinal cord injury but had a negative effect on hippocampal neurodegeneration. For example, Hall (J. Neurosurg vol. 76, 13-22 (1992)) noted that the glucocorticoid steroid, methylprednisolone, believed to involve the inhibition of oxygen free radical-induced lipid peroxidation, could improve the 6-month recovery of patients with spinal cord injury when administered in an intensive 24-hour intravenous regimen beginning within 8 hours after injury. However, when the steroid was examined for selective protection of neuronal necrosis of hippocampal neurons, it was found that the hippocampal neuronal loss was significantly worsened by glucocorticoid steroid dosing suggesting that this hormone is unsuitable for treating acute cerebral ischemic. Hall reported that substitution of a complex amine on a non-glucocorticoid steroid in place of the 21'-hydroxyl functionality results in an enhancement of lipid anti-oxidant activity. No data were provided concerning the behavior of this molecule in treating ischemic events or in neuroprotection of neurons in the brain. Additionally, free radical scavenging activity has been reported for a lazaroid, another non-glucocorticoid steroid having a substituted 21'-hydroxyl functionality, but there is no evidence that this compound is significantly efficacious for treating stroke or other forms of ischemia.

Summary

The invention satisfies the above need. Novel methods are provided for prevention and treatment of ischemic damage using estrogen compounds.

A preferred embodiment of the invention provides a method for conferring protection on a population of cells associated with an ischemic focus, in a subject following an ischemic event that includes the steps of providing subcutaneously an

estrogen compound in a drug delivery system in which the estrogen compound is dissolved in oil with or without additional excipients such as solvents, stabilizers or preservatives, so as to confer protection on the population of cells. Further embodiments include selecting a proximate time for administering the effective dose of the estrogen compound that is prior to the ischemic event. Alternatively, the estrogen compound may be administered within an effective proximate time after the ischemic event. The method of the invention may be applied to any of a cerebrovascular disease, subarachnoid hemorrhage, myocardial infarct, surgery, and trauma. In particular, when the ischemic event is a stroke, the protected cells include at least one of neurons and endothelial cells.

The method utilizes an estrogen compound that may include alpha isomers or beta isomers of estrogen compounds. Examples of different isomers are provided wherein the estrogen compound is selected from the group consisting of 17α-estradiol and 17β-estradiol.

In a preferred embodiment of the invention, a method is provided for protecting cells in a subject from degeneration during or after an ischemic event. The steps of the method include identifying a susceptible subject, providing an effective dose of an estrogen compound prior to or after the ischemic event, and protecting cells from degeneration otherwise occurring in the absence of the estrogen compound.

In a further embodiment of the invention, a method is provided for treating stroke in a subject, including the steps of providing an effective dose of an estrogen compound in a pharmaceutical formulation and administering the formulation to the subject so as to reduce the adverse effects of the stroke.

The invention in another embodiment provides a method for conferring protection on a population of cells associated with ischemia, in a subject following an ischemic event, comprising: (a) providing an estrogen compound formulated in an oil vehicle; and (b) administering an effective amount of the compound over a course that includes at least one dose within a time that is effectively proximate to the ischemic event, so as to confer protection on the population of cells. Further in this embodiment in (b) the estrogen compound is administered by subcutaneous injection.

In another embodiment, the invention provides a method of synthesis of *ent*-17β-estradiol from [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one,

comprising: reducing the double bond of [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one to obtain tricyclic compound [3*R*-(3α,3aα,5aβ,6β,9aα,9bβ)]-3-(1,1-dimethylethoxy)-dodecahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one: cyclizing the tricyclic compound [3*R*-(3α,3aα,5aβ,6β,9aα,9bβ)]-3-(1,1-dimethylethoxy)-dodecahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one to obtain *ent*-19-nortestosterone; esterifying the hydroxy group of *ent*-19-nortestosterone to obtain *ent*-19-nortestosterone, 17-acetate; aromatizing the steroid A ring of *ent*-19-nortestosterone, 17-acetate to obtain *ent*-17β-estradiol, 17-acetate; and saponifying *ent*-17β-estradiol,17-acetate to remove the 17-acetate group, to obtain *ent*-17β-estradiol.

According to this method, reducing the double bond of [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one is obtained by a step selected from the group consisting of: using lithium in liquid ammonia, and using catalytic hydrogenation.

Another embodiment of the invention provides the compound [3*R*-(3α,3aα,5aβ,6β,9aα,9bβ)]-3-(1,1-dimethylethoxy)-dodecahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one.

A further embodiment of the invention provides the compound *ent*-17β-estradiol, 17-acetate. Yet another embodiment of the invention is the compound *ent*-19-nortestosterone, 17-acetate.

Brief Description of the Drawings

These and other features, aspects, and advantages of the present invention will be better understood with reference to the following description, appended claims, and accompanying drawings, where:

Fig 1. is a bar graph that shows the effects of pretreatment of ovariectomized rats, with 17β-estradiol, initiated 24 hours prior to ischemia induced by middle cerebral artery occlusion (MCAO); where the 17β-estradiol is administered as a subcutaneous 5mm Silastic® implant (E2) or via the estradiol-chemical delivery system (E2-CDS) (1mg/kg body weight) and a control is provided (a sham pellet). Values are given as the mean plus and minus the standard error of the mean (± SEM) for the percent ischemic area in 3 brain

slices. The asterisk indicates that the observed p value was less than 0.05 (*=p<0.05) vs. sham group, that is, that the difference between the data for the experimental group and the sham group was statistically significant. The number of samples for sham=6, for 17β-estradiol=8, and for E2-CDS groups=10.

Fig 2. is a bar graph that shows the effects of treatment of ovariectomized (OVX) rats with 17β-estradiol, at 2 hours prior to ischemia induced by MCAO, where the 17β-estradiol (10μg/kg) is injected subcutaneously in an oil vehicle. Rats were decapitated 24 hours after the MCAO. Rat brains were dissected coronally as region A- E, 24 hours after MCAO. Values were given as the mean ± SEM where n=8 for OVX + E2 group and n=6 for OVX group(control). *p<0.05 vs. corresponding vehicle control groups.

Fig. 3 is a bar graph that shows the effects of pretreatment of ovariectomized rats with 17α-estradiol, initiated 24 hours prior to ischemia induced by MCAO, where the 17α-estradiol is administered in a 5mm Silastic® tube, and the negative control is a 5mm Silastic® tube without estrogen (sham). Rats were decapitated 24 hours after the MCAO. Values are given as the mean ± SEM for the percent ischemic area in 5 brain slices. A to E designate the distance caudal to the olfactory bulb A=5 mm, B=7 mm, C=9 mm, D=11 mm, and E = 13 mm. *= p<0.05 vs. sham group for the equivalent brain slice; for sham n = 10 and for 17α-estradiol groups, n=13.

Fig. 4 is a bar graph that shows the effects of post-treatment of ovariectomized rats with 17β-estradiol or an hydroxypropyl cyclodextrin (HPCD) control at 40 minutes (a) and 90 minutes (b) post onset of MCAO. The 17β -estradiol was formulated in an estradiol chemical delivery system (E2-CDS) at a concentration of 1mg/kg body weight and injected intravenously. Rats were decapitated 24 hours after the MCAO. Values are given as the mean ±SEM for the percent ischemic area in 5 brain slices. A to E designate the distance caudal to the olfactory bulb A=5 mm, B=7 mm, C=9 mm, D = 11 mm and E = 13 mm. Where *=p<0.05 vs HPCD group for the same brain slice, N=9 for vehicle, and 13 for E2-CDS groups.

Fig. 5 is a bar graph that shows the effects of 17β-estradiol (2nM) on brain capillary endothelial cell (BCEC) mortality following 24 hours of hypoglycemia. The control consists of the ethanol vehicle only. The glucose concentrations in the cell media were adjusted from 20mg% to 200mg% by adding appropriate amount of D-(+)-glucose to the glucose-free media. BCEC were incubated for 24 hours (a) and 48 hours (b).

Trypan blue staining was used to distinguish live cells from dead cells. Two cell countings at two different hemacytometer squares were averaged. Mean ± SEM are depicted (n=8-12). *p<0.05 vs. corresponding vehicle control.

Fig. 6 is a bar graph that shows the effects of 17β-estradiol (2nm) on BCEC mortality following anoxia. The control consists of the ethanol vehicle without estrogen. Cell media contained 200 mg% glucose. Culture dishes containing BCEC were placed in nitrogen filled chamber for 4 hours. Trypan blue staining was used to distinguish live cells from dead cells. Two cell countings at two different hemacytometer squares were averaged. Mean ± SEM are depicted (n=8-12). *p<0.05 vs. corresponding vehicle control.

Fig. 7 is a bar graph that shows the effects of 17β-estradiol (2nm) on BCEC mortality compared with a control (ethanol vehicle) following a combination treatment of both anoxia and hypoglycemia. Cell media contained 200 mg% or 100 mg% glucose. Culture dishes containing BCEC were placed in either an incubator or a nitrogen filled chamber for two hours. Trypan blue staining was used to distinguish live cells from dead cells. Two cell countings at two different hemacytometer squares were averaged. Mean ± SEM are depicted (n=8.12). *<0.05 vs. corresponding vehicle control.

Fig. 8 is a bar graph that shows the effects of post-treatment of ovariectomized (OVX) rats with 17β-estradiol at 0.5 hour, 1 hour, 2 hours, 3 hours or 4 hours following ischemic induced by MCAO. The estrogen compound was administered by a combination of an intravenous preparation (100 μg/kg) of HPCD-complexed 17β-estradiol and Silastic® pellet at the times post-occlusion indicated. Ovariectomized, non-treated animals(OVX) and non-ovariectomized, non-treated animals (INT) were used as controls (n=12 and n=6, respectively). At 48 hours following MCAO, ischemic lesion volume was determined using 2,3,5 - triphenyltetrazolium (TTC) staining.

Fig. 9 is a graph that shows the effects on drug kinetics of administering an estrogen compound in single subcutaneous bolus injection in oil on the ordinate, as a function of time on the abscissa.

Figure 10. Structure of the naturally occurring 17β-estradiol (βE2) and the non-naturally occurring *ent*-17β-estradiol (*ent*-E2).

Figure 11. Effects of 17β-estradiol (βE2) and *ent*-E2 on glutamate toxicity in the HT-22 cells. The indicated concentration of steroid was added 2 hours prior to the

addition of glutamate (5mM) and viability was assessed 24 hours later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 100% viability and mean ± sem is shown for the average of 2 experiments with 4-8 wells per experiment. *=$p<0.05$ and **=$p<0.01$ versus toxin only group. Pictured are representative fields stained with calcein AM and propidium iodide.

Figure 12. Effects of 17β-estradiol (βE2) and *ent*-E2 on H_2O_2 toxicity in HT-22 cells. 10nM of the steroid was added to HT-22 cells 2 hours prior to the addition of the indicated concentration of H_2O_2. Viability was assessed 24 hours later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 0% reduction in viability and shown as mean ± sem for the average of 2 experiments with 4 wells per experiment. *=$p<0.05$ versus toxin only group.

Figure 13. Effect of *ent*-E2 on H_2O_2 toxicity in SK-N-SH cells. The indicated concentration of *ent*-E2 was added 24 hours prior to the addition of 3μM H_2O_2. Viability was assessed 24 hours later using calcein AM fluorescence. Relative fluorescence units were normalized to the respective toxin-free group as 100% viability and shown as mean ± sem for the average of 2 experiments with 3-4 wells per experiment. *=$p<0.05$ and **=$p<0.001$ versus the toxin only group.

Figure 14. Effects of 17β-estradiol (βE2) and *ent*-E2 on MCA occlusion-induced lesion volume in ovariectomized female rats. Rats were ovariectomized 2 weeks prior to occlusion and steroids were administered by subcutaneous injection 2 hours prior to onset of focal ischemia. Following 1 hour MCA occlusion and 23 hours reperfusion, the brains were removed and 2 mm slices prepared at 3, 5, 7, 9, and 11 posterior to the olfactory bulb. Lesion volume was determined by TCC staining. Graphed is mean ± sem for 6 rats per group. *=$p<0.05$ versus vehicle treated rats. Pictured are representative slices for each treatment group.

Figure 15. Plasma 17β-estradiol (βE2) levels following 17β-estradiol (βE2) and *ent*-E2 administration. Ovariectomized female Sprague-Dawley rats were injected subcutaneously with either 100 μg/kg 17β-estradiol (βE2) or *ent*-E2. Blood was drawn by cardiac puncture either 5 minutes prior to injection, 2 hours post injection, 4 hours post injection, or 24 hours post injection. Plasma was collected and 17β-estradiol (βE2) concentration determined by RIA. The mean for 3 ± sem rats per group is shown.

Figure 16. Effects of 17β-estradiol (βE2) and *ent*-E2 on uterine weight in juvenile rats. 25 day old female rats were injected subcutaneously with the indicated dose of 17β-estradiol (βE2) or *ent*-E2, or concurrent administration of the indicated dose of *ent*-E2 with 1 μg/kg 17β-estradiol (βE2) daily for3 days. On day 4, the uteri were resected and weighed. Shown are mean ± sem for 3 to 9 rats per group. *=$p<0.05$ versus oil injection.

Figure 17. 17β-estradiol (βE2) and *ent*-E2 inhibit $FeSO_4$-induced lipid oxidation in a rat brain homogenate. Homogenate was prepared from the neocortical tissue of an ovariectomized female Sprague-Dawley rat. Homogenate was incubated with the indicated concentraton of steroid for 30 minutes, and then oxidized by a 30 minute incubation with 200 μM $FeSO_4$ at 37°C. The extent of lipid oxidation was determined by TBAR formation. Data were normalized to $FeSO_4$ only group as 100% oxidation. Shown are mean ± sem for 3 samples per group. *=$p<0.05$ versus $FeSO_4$ only group.

Detailed Description of Specific Embodiments

There is a need for effective treatments for stroke and other forms of ischemia that are safe, and may be administered preventatively to men and women who are susceptible to such conditions, and may further be used after the ischemia has occurred so as to protect cells from progressive degeneration that is initiated by the ischemic event. There is further a need for therapeutic strategies, to treat victims of stroke or other forms of ischemic events such as myocardial infarction, in which the active drug could enter the bloodstream very rapidly, reach peak levels within minutes, and sustain lower, therapeutic drug dosage levels for a significant period of time (e.g., hours) thereafter.

The invention provides an effective treatment for stroke and other forms of ischemia that may safely be administered to men and women so as to protect cells from progressive degeneration that is initiated by the ischemic event.

Estrogen compounds are defined here and in the claims as any of the structures described in the 11th edition of "Steroids" from Steraloid Inc., Wilton, N.H., incorporated herein by reference. Included in this definition are non-steroidal estrogens described in the aforementioned reference. Other estrogens included in this definition are estrogen derivatives, estrogen metabolites, estrogen precursors, and modifications of the foregoing as well as molecules capable of binding cell associated estrogen receptor as well as other molecules where the result of binding triggers a characteristic estrogen effect. Any

diastereomer or enantiomer of compounds described herein is included in the definitions herein. Also included are mixtures of more then one estrogen. The term "estradiol" or "estrogen" is included in the meaning of estrogen compound.

β-estrogen and α-estrogen are isomers of estrogen.

The term "E2" is synonymous with β-estradiol,17β-estradiol, E_2, and β-E_2.

An "animal subject" is defined here and in the claims is a higher organism including a human subject.

The term "non-sex hormone" is defined here and in the claims as an estrogen compound having diminished, minimal or no sex-related effect on the subject.

Estrogen compounds are here shown to protect cells from degeneration in the penumbra of the ischemic lesion (Examples 1 and 2). Estrogen compounds are further shown to be protective of a plurality of cell types, including neuronal cells and endothelial cells (Examples 1-3). According to the invention, estrogen compounds may be used to protect cells from the effects of oxygen deprivation and glucose deprivation and consequently from energy deprivation associated with ischemia.

In an embodiment of the invention, a method of treatment is provided that is suitable for human male and female subjects and involves administering an effective dose of estrogen either before or after a stroke has occurred.

In certain circumstances according to the invention, it is desirable to administer estrogen prior to a predicted ischemic event. Such circumstances arise when, for example, a subject has already experienced a stroke. In this case, the subject will have an increased probability of experiencing a second stroke. Subjects who are susceptible to transient ischemic attacks also have an increased risk of a stroke. Subjects who suffer a subarachnoid hemorrhage may experience further ischemic events induced by vasospasms that constrict the blood vessels. Subjects who experience trauma to organs such as the brain are also susceptible to an ischemic event. The above situations exemplify circumstances when a subject would benefit from pretreatment with an estrogen compound. Such pretreatment may be beneficial in reducing the adverse effects of a future ischemic event when administered in the short term, such as within 24 hours before the event (Example 1) or in the long term, where administration begins immediately after an event such as a stroke and continues prophylactically for an extended period of time. An example of time of administration for prophylactic use may extend from days to

months depending of the particular susceptibility profile of the individual. In these circumstances, a course of at least one dose of estrogen may be administered over time so that an effective dose is maintained in the subject. For short term treatments, parenteral administration may be used as an alternative to the delivery of a dose by any of the routes specified below. The optimal dose of estrogen compound for prophylactic use should provide a plasma concentration of 10-500 pg/ml of estrogen compound, however higher doses are also acceptable. In these circumstances, the use of non-sex estrogen compounds such as the α-estrogen isomers are of particular utility in men and women because the sex-related functions of the hormone are avoided.

According to embodiments of the invention, estrogen compounds are effective in reducing the adverse effects of an ischemic event such as cerebrovascular disease, subarachnoid hemorrhage, or trauma. Accordingly, the compound is administered as soon as possible after initiation of the event and preferably within 12 hours, more particularly, within 5 hours following the event. It is desirable that an increased concentration of estrogen compound be maintained in the plasma for at least several hours to several days following the ischemic event. The increased concentration of estrogen compound in the plasma should be in the range of 10-12,000 pg/ml of estrogen compound.

The present invention demonstrates for the first time that pretreatment with estrogens or early post-treatment of an estrogen compound can significantly reduce the size of the necrotic area following an ischemic event. This effect of pretreatment with an estrogen compound is independent of the isomeric form and the route of administration of the estrogen compound. α-isomers of estrogen have been shown to be as effective as β-isomers of estrogen in protecting cells from the effects of ischemia. The method as exemplified in Example 1 and Figs. 1, 2 and 3 confirm that the protective activity of estrogen compounds is not dependent on the sex-related activity of the hormone (estrogenicity). α-isomers of estrogen compounds are non-sex hormones, yet these compounds are as effective at protecting the brain against ischemic damage as the β-isomers. Example 1 further demonstrates that the observed reduction in mortality of ovariectomized rats when treated with 17β-estradiol is not dependent on the route of administration, since the protective effect was similar when the same estrogen compound was administered as a subcutaneous implant or as an intravenous injection. Regardless

of the route of administration or the formulation, the estrogen compounds have a remarkable effect on the ability of animals to survive an ischemic event.

The demonstration that estrogen is efficacious in protection of cells in an ischemic area is demonstrated in the examples below using rat models in which the middle cerebral artery (MCA) is experimentally occluded, the middle cerebral artery occlusion (MCAO) model. This animal model is well known in the art to simulate an *in vivo* ischemic event such as may occur in a human subject. The experimental occlusion of the MCA causes a large unilateral ischemic area that typically involves the basal ganglion and frontal, parietal, and temporal cortical areas (Menzies et al. Neurosurgery 31, 100-106 (1992)). The ischemic lesion begins with a smaller core at the site perfused by the MCA and grows with time. This penumbral area around the core infarct is believed to result from a propagation of the lesion from the core outward to tissue that remains perfused by collateral circulation during the occlusion. The effect of a therapeutic agent on the penumbra surrounding the core of the ischemic event may be examined when brain slices are obtained from the animal. The MCA supplies blood to the cortical surfaces of frontal, parietal, and temporal lobes as well as basal ganglia and internal capsule. Slices of the brain are taken around the region where the greatest ischemic effect occurs. These regions have been identified as region B, C, and D in Examples 2 and 3. These regions are not as readily compensated by alternative sources of blood flow as are regions A and E. This is because the MCA is the terminal artery on which the lace of collateral arteries supplying the MCA-distributed area relies, thereby making the MCA-occlusion induced ischemia uncompensatible. On the other hand, anastomoses between MCA and the anterior carotid artery (ACA) in region A and between MCA and the posterior carotid artery (PCA) in region E (Examples 1 and 2), may compensate for the MCA occlusion-induced ischemia as observed in the present study.

In order to study the effect of estrogen on the propagation of the lesion following an ischemic event, rats were ovariectomized and two weeks later were exposed to various estrogen preparations prior to or following MCAO. (Examples 1 and 2). Untreated, ovariectomized rats had a mortality of 65%. Pretreatment with E2-CDS or 17β-estradiol itself decreased mortality from 16% and 22%, respectively. This marked reduction in mortality was accompanied by a reduction in the ischemic area of the brain from 25.6±5.7% in the untreated, ovariectomized rats to 9.1±4.2% and 9.8±4.0 in the E2-CDS

or 7β-estradiol treated rats, respectively. Similarly, pretreatment with non-sex hormones, exemplified by 17α-estradiol, reduced ischemic area by 55 to 81% (Example 1). When administered 40 or 90 minutes after MCAO, 17β-estradiol reduced ischemic area by 45-90% or 31%, respectively (Example 2). Non-sex hormones were also highly protective when administered following induction of ischemia. These results demonstrate the neuroprotective effect of estrogen compounds in the brain following an ischemic event.

Reduction in available oxygen and glucose for energy metabolism is a feature of an ischemic event. This has a negative impact on the blood vessels that may be required to supply nutrients once the occlusion is reversed. The negative effect on blood vessels following ischemia further increases the long-term damage associated with the event. This effect can be reproduced *in vitro* as described in Example 3. In these circumstances, it has been shown here, estrogen compounds are capable of protecting brain capillary endothelial cells from cell death that would otherwise occur during hypoglycemia and anoxia during an ischemic event (Figs. 5-7). As a consequence of this protection, the integrity of the vascular supply and the blood brain barrier is preserved by estrogen compounds such that following reperfusion of the brain after the ischemic event, blood flow and transport functions can once again occur.

Estrogen compounds are shown here to be effectively delivered subcutaneously in an oil vehicle (Example 5 and Fig. 9). This mode of delivery was successful at achieving blood levels of 4,610 pg/ml of the estrogen compound within 30 minutes. Sustained delivery was achieved also, as animal blood levels of 2,004 pg/ml was at the four hour time point (Fig. 9).

Synthesis of *ent*-17β-estradiol is shown by the methods of Example 6, and in Table 3. Fig. 10 and Example 7 shows that *ent*-17β-estradiol was as effective a therapeutic agent as *ent*-17β-estradiol.

EXAMPLES

Example 1. Measurement of the effect of estrogen compound administered prior to ischemic events.

Rats were used as experimental models to test the effects of estrogen compounds in protecting against ischemic damage. To remove the naturally occurring source of estrogen, ovariectomies were performed prior to induction of ischemia.

Subsequent to the ovariectomy, rats were treated with an estrogen compound either by subcutaneous delivery with Silastic® tubes 24 hours prior to the MCA occlusion or by intravenous delivery as follows:

Subcutaneous sustained delivery: 17β- or 17α-estradiol was packed into 5mm long Silastic® tubes (Dow-Corning, Midland, MI) according to the method of Mohammed et al. 1985 Ann. Neurol 18, 705-711. Sham (empty) tubes were similarly prepared as estrogen negative controls. The pellets were implanted subcutaneously (sc) into ovariectomized rats 24 hours prior to MCAO. 5mm of Silastic® tubing containing estrogen resulted in plasma levels of about 100-200pg/ml.

Intravenous (iv) delivery: 17β-estradiol was prepared for iv delivery using an estrogen-chemical delivery system (E2-CDS) as described in Brewster et al., Reviews in the Neurosciences 2, 241-285 (1990) and Estes et al., Life Sciences 40: 1327-1334 (1987). E2-CDS was complexed with hydroxypropyl- -cyclodextrin (HPCD) (Brewster et al. J. Parenteral Science and Technology 43: 231-240, (1989)). The complexation achieved was 32 mg of E2-CDS per gram HPCD. In the first study, a single iv injection of E2-CDS (1mg/kg body weight) was administered at 24 hours prior to MCAO. The control was administered HPCD only. The chemical delivery system is formulated so that the estrogen is slowly released from the carrier. This delivery system has been shown to effectively deliver estrogen in a sustained manner to the brain. Indeed, the dose of E2-CDS used in Examples 1 and 2 (1mg/kg) is sufficient to provide 1000 pg/gm brain tissue at 24 hours post administration.

At 7 to 8 days after ovariectomy, a method for occluding the middle carotid artery was applied to the rat using modifications of the methods of Longa et al. (1989) Stroke, vol. 20, 84-91; and Nagasawa et al. (1989); Stroke, vol. 20, 1037-1043, with certain modifications, as described herein.

Animals were anesthetized by ip injection with ketamine (60 mg/kg) and xylazine (10 mg/kg). Rectal temperature was monitored and maintained between 36.5 and 37.0 C with a heat lamp throughout the entire procedure. The left carotid artery was exposed through a midline cervical incision. The left sternohyloid, sternomastoid, digastric (posterior belly) and the omohyloid muscles were divided and retracted. Part of the greater horn of the hyloid bone was cut to facilitate exposure of the distal external carotid artery (ECA). The common carotid artery (CCA), ECA, and internal carotid artery (ICA)

were dissected away from adjacent nerves. The distal ECA and its branches, the CCA, and the pterygopalatine arteries were coagulated completely. A microvascular clip was placed on the ICA near skull base. A 2.5 cm length of 3-0 monofilament nylon suture was heated to create a globule for easy movement and blocking of the lumen of the vessel. This was introduced into the ECA lumen through the puncture. The suture was gently advanced to the distal ICA until it reached the clipped position. The microvascular clip was then removed and the suture was inserted until resistance was felt. The distance between the CCA bifurcation and the resistive point was about 1.8 cm. This operative procedure was completed within 10 minutes without bleeding. After the prescribed occlusion time (40 minutes), the suture was withdrawn from the ICA and the distal ICA was immediately cauterized.

Animals that survived until the scheduled sacrifice time were sacrificed by decapitation. Scheduled post-ischemic sacrifices occurred at 6 hours, 24 hours and 1 week post MCAO (Table 1). For the 6-hour sample, animals were monitored continuously. For the 24-hour sample, animals were observed for about 4 hours and were then returned to their cages. Similarly, animals scheduled for the 1 week post-ischemic sacrifice were monitored for the first 4 hours after surgery and then daily thereafter.

The brains were isolated from the decapitated heads, sliced into 3 or 5 coronal tissue slices as described below and then stained with hematoxylin and eosin to determine the extent of the ischemic area. Stained slices were photographed and subsequently imaged using a Macintosh Cadre 800 computer, equipped with an Image 1.47 software program for the assessment of the cross-sectional area of the ischemic lesion. These images and the calculated area of ischemic damage were stored in the program for later retrieval and data reduction. The significance of differences in mortality among the different treatment groups was determined using Chi-Square analysis.

The results obtained using different routes of administration and different isomeric forms of estrogen compounds are provided below.

The administration of an estrogen compound by subcutaneously using Silastic® tubes or by controlled intravenous delivery, at 24 hours prior to the ischemic event, caused brain lesion size and mortality to be reduced.

Three coronal slices were made at 1, 5, and 7mm posterior to the olfactory bulb. Only 35% of the control (sham) animals survived until the scheduled post-ischemic

sacrifice time (Table 1). In contrast, 78% and 84% of animals, treated 24 hours prior to MCAO with either 17β-estradiol in a Silastic® tube (E2 implant) or with E2-CDS at 1mg/kg administered by iv injection survived until the scheduled post-ischemic sacrifice time at 6 hours, 1 day, and 1 week. Elevated levels of 17β-estradiol were detected in all samples at the time of sacrifice. The reduction in mortality in the estrogen compound pretreatment group was most notable at 1 day and 1 week after MCAO (Table 1). Furthermore, the reduced mortality in the estrogen compound treated rats was correlated with the reduction of ischemic area in animals that survived to the scheduled 1 day or 1 week post-ischemic sacrifice time (Fig. 1). Control (sham) rats had ischemic lesions that occupied 25.6 ± 5.7% of the cross-sectional area of brain sections evaluated (Fig. 1). By contrast, rats treated with 17β-estradiol in Silastic® tubes or E2-CDS had ischemic lesions that occupied only 9.8±4.0 and 9.1± 4.2%, respectively, of the brain area evaluated. The significance of differences among groups was determined by analysis of variance (ANOVA) and the Fischer's test was used for the post hoc comparison. Determination of areas under the curves were not done here as only three brain slices were taken.

The results shown in Fig. 2 illustrate the significant protective effect of estrogen compounds in tissue slices A-D in animals treated with subcutaneous injection of 17β-estradiol (10μg/ml) two hours prior to an ischemic event.

Rats were ovariectomized, treated with a single dose of 17β-estradiol (10μg/kg) administered by sc injection, 14 days after the ovariectomy and two hours prior to the ischemic event as described above. This injection was sufficient to achieve a plasma concentration of 250pg/ml at the time of occlusion. The animals were sacrificed at 24 hours and the brains extracted. Estrogen compound replacement of ovariectomized rats reduced by 46.3% and 44.1% ($p<0.05$) ischemic lesion size of the whole coronal section at region C and D, respectively (Fig. 2). These regions correspond to sections taken at 9 and 11 mm caudal to the olfactory bulb.

The results shown in Fig. 3 illustrate the significant protective effect of 17α-estradiol in tissue slices A-E in animals treated with a sustained subcutaneous delivery of 17α-estradiol initiated 24 hours prior to the ischemic event.

Ovariectomized rats were treated with 5mm Silastic® tubes containing 17α-estradiol at 24 hours prior to MCAO. At 24 hours after the MCAO, the animals were sacrificed and the brains extracted. Five, 2mm thick coronal sections were made at 5, 7,

9, 11, and 13 mm posterior of the olfactory bulb. The slices were then incubated for 30 minutes in a 2% solution of 2,3,5-triphenyl tetrazolium (TTC; Sigma Chemical Corp., St. Louis, MO) in physiological saline at 37 C. Sham-treated rats showed the expected ischemic lesion, with the maximum ischemic area (24.1±2.4%) occurring in slice C (9mm posterior to the olfactory bulb) and smaller lesion areas occurring in more rostral and caudal slices (Fig. 3). The significance of differences between sham and steroid-treated groups, were thus determined and data from two groups were compared for each experiment. To determine the area under the lesion curve for a given treatment, the trapezoidal method was used. Areas calculated for each animal were grouped and the differences between groups were determined by the student t test.

Animals pretreated with 17α-estradiol exhibited smaller ischemic areas compared with the sham treated animals in all slices evaluated (Fig. 3, A-E). Specifically, slices C, D and E (sections taken at 7, 9, and 11 mm posterior to the olfactory bulb), ischemic area was reduced significantly by 55%, 66%, and 81%, respectively (Fig. 3). The area under the ischemic lesion curve for the sham-treated, and the 17α-estradiol groups was 8.1±0.8 and 3.7±1.3, respectively (Table 2).

Example 2. Measurement of the effect of estrogen compounds administered after the ischemic event.

To test the extent to which estrogen treatment was effective after the onset of the occlusion, ovariectomized rats were treated iv with a sustained release of either E2-CDS or with a control (HPCD vehicle), the positive sample causing a brain tissue concentration of estrogen of 1000pg estrogen/gm brain tissue, 24 hours after administration. The estrogen compound was administered at 40 minutes and 90 minutes after the onset of the MCAO (Fig. 4a and b, Table 2) and the animals sacrificed at 24 hours after the MCAO. Five 2 mm thick coronal sections were made at 5, 7, 9, 11, and 13 mm posterior of the olfactory bulb as described in Example 1.

Post-treatment at 40 minutes: As shown in Figure 4a, the control rats (HPCD treated) had large ischemic areas in all slices sampled, with the maximum ischemic area of 25.6±2.7% observed in slice C. E2-CDS treatment reduced ischemic area in all slices sampled (Fig 4). The extent of reduction in ischemic area ranged from 90% in slice A (5mm posterior of the olfactory bulb) to 45% in slice C (9m posterior to the olfactory

bulb) (Fig 4a). The integrated area under the ischemic lesion curve was 10.1±1.6 for the vehicle treated rats and 4.5±0.9 for the E2-CDS animals (Table 2).

Post-treatment at 90 minutes: Rats were treated with E2-CDS or HPCD vehicle at 90 minutes after the onset of the occlusion (Fig. 4b and Table 2). Again, HPCD treated animals showed a large lesion in all slices sampled, with the maximum ischemic area seen in slice C (20.5±3.1% of the slice area). Treatment with E2-CDS reduced the mean ischemic area in all slices examined, however, the differences were not statistically significant. An evaluation of the area under the ischemic curve for the two groups revealed that treatment with E2-CDS reduced the ischemic area by 37.1%, from 8.2±1.7 (HPCD treated animals) to 5.2±1.7 (E2-CDS treated animals).

Example 3. Estrogen compounds protect brain capillary endothelial cells under conditions associated with focal ischemia.

Primary rat brain capillary endothelial cells (BCEC) cultures were prepared following the method of Goldstein, J. Neurochemistry vol. 25, 715-717, 1975, incorporated herein by reference.

Hypoglycemia experiments were undertaken. 17β-estradiol (2nm) or control (ethanol vehicle) were added to BCEC cultures. The glucose concentration of the culture media was then adjusted from 20mg% to 200mg% by adding appropriate amount of D-(+)-glucose to the glucose-free media and monitored by Glucose and L-Lactate Analyzer (YSI model 2300 STAT plus, YSI, Inc., Yellow Springs, OH). The hypoglycemic cultures were maintained for 24 hours or 48 hours prior to staining with Trypan blue.

Anoxia environment was created by placing culture dishes containing BCEC with or without 2mm 17β-estradiol in the Modular Incubator Chamber (Billups-Rothenberg, Inc., Delmar, CA). Nitrogen gas was influxed to replace the oxygen inside the chamber. The chamber was sealed and placed in the incubator for four hours for nonhypoglycemic cultures and 2 hours for hypoglycemic cultures.

Cell mortality was counted using Trypan blue staining method. Cell death percentage was calculated as dead cell/alive cell x100%.

Statistical methods used included two-way analysis of variance, applied to determine the significance of the difference among the experimental groups. Kruskal-Wallis nonparametric analysis was used for data presented as percentage. The Mann-

Whitney U tests were used when Kruskal-Wallis showed significance among groups. $P<0.05$ was considered significant.

The results are shown in Fig 5a and 5b for cells deprived of glucose. The normal glucose concentration in the media is 200 mg per 100 ml (200 mg%). Little difference was observed in percent cell death between cultures with and without estrogen supplement at this glucose concentration. However, reduction in medium glucose content to 100 mg%, 40mg%, and 20mg% caused cell death, and 17β-estradiol saved cell loss by 35.9%, 28.4% and 23.% ($p<0.05$), respectively, compared with corresponding control groups not exposed to the estrogen compound. It was further noted that there were floating cells, which meant more dead cells, in the control groups than in the estradiol-treated groups. Since these cells were excluded when counting cell mortality, the protective effects of estradiol may be underestimated. A similar beneficial effect was observed over a 24 hour and 48 hour hypoglycemic treatment (Fig. 5a and b, respectively).

Anoxia had a more dramatic effect in cell viability as shown in Fig. 6 for cells in media containing 200mg% glucose. Anoxia induced cell death as much as 48.8% and 39.8% in the control and E2 reduced cell death by 28.4% ($p<0.05$) at 1 hour and 18.4% ($p<0.05$) at 4 hour anoxic insults.

When cells were exposed to both hypoglycemia (100 mg% hypoglycemia) and anoxia conditions (2 hours), 17β-estradiol was effective in protecting cultured BCEC from the cumulative effect of both conditions (Fig. 7).

The *in vitro* assay is representative of events that follow ischemia such as that induced by MCAO where oxygen and glucose supplies to the of the blood brain barrier endothelial cells are reduced.

Example 4. Comparison of post-treatment at 0.5, 1, 2, 3 and 4 hour time points.

Ovariectomized rats were treated with both an iv injection (100 μg/kg) of HPCD-complexed 17β-estradiol and a 17β-estradiol containing Silastic® pellet at the times indicated after the onset of occlusion (Fig. 8). HPCD and HPCD-encapsulated 17β-estradiol were purchased from Sigma (St. Louis, MO). Ovariectomized, non-treated animals (OVX) and non-ovariectomized, non-treated animals (INT) were used as controls ($n=12$ and $n=6$, respectively). At 48 hours following MCAO, animals were sacrificed and ischemic lesion volume was determined by obtaining brain sections as previously

described and staining with TTC. Fig. 8 shows that significant protection was observed when drugs were administered at 0.5, 1, 2, or 3 hours post-occlusion.

Example 5. Delivery of an estrogen compound using an oil vehicle.

To test the kinetics of uptake of an estrogen compound in an oil vehicle, male Sprague-Dawley rats (Taconic) were given 17β-estradiol by sc injection, and drug levels in the blood were determined over a 25 hour period. The drug was dissolved in corn oil at 100 μg/ml and the final dosage delivered was 100 μg/kg. Blood samples were drawn at 30 minutes prior to drug administration, 30 minutes after drug administration, 4 hours after drug administration and 24 hours after drug administration. Venous blood was collected into heparinized tubes, centrifuged and the plasma was collected and frozen. Levels of 17β-estradiol were determined using a commercially supplied radioimmunoassay kit.

As shown in Fig. 9, there was a significant, very rapid uptake of the 17β-estradiol into the bloodstream, peaking in this experiment at the 30 minute time point (at 4,610 pg/ml). At 4 hours, the level of circulating 17β-estradiol was 2,004 pg/ml. By 25 hours, 17β-estradiol blood levels had fallen off to near zero.

These delivery kinetics indicate that the delivery vehicle described here in which the estrogen compound was dissolved in oil and delivered by a single subcutaneous injection into animals serves the dual purpose of initiating rapid uptake of the compound into the blood, and providing for sustained delivery of the compound for hours thereafter.

Example 6. Synthesis of *ent*-17β-estradiol.

The synthesis of *ent*-17β-estradiol is summarized in Table 3. The known starting material, [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one (Chemical Abstracts Registry Number, 139973-49-2), was prepared by a multistep synthetic pathway as described in the literature (Rychnovsky, S.D. et al. J. Org. Chem. 1992 vol.57, 2743-2736). This compound was then converted in either of two ways (Method A or Method B) to *ent*-19-nortestosterone (Chemical Abstracts Registry Number, 4091-86-5).

In the first step of Method A, the double bond is reduced using lithium in liquid ammonia and the resulting tricyclic compound is cyclized to *ent*-19-nortestosterone in the second step. In the first step of Method B, the double bond is reduced by catalytic

hydrogenation and the resulting tricyclic compound is again cyclized to *ent*-19-nortestosterone in the second step. Method B has been previously used to prepare 19-nortestosterone (Micheli, R.A. et al.. 1975 J.Org.Chem. Vol.40, 675-681). The hydroxy group of *ent*-19-nortestosterone is then esterified and the A-ring of the steroid is aromatized using $CuBr_2$ in acetonitrile. This reaction has been reported previously for the conversion of 19-nortestosterone,17-acetate to 17β-estradiol,17-acetate (Rao, P.N. et al. 1994, Steroids vol.59, 621-627). Finally, the 17-acetate group is removed by saponification to give

Ent-17β-estradiol (Chemistry Abstracts Registry Number, 3736-22-9). The structure of *Ent*-17β-estradiol was proven by experimental data, which showed that the compound had the same melting point, IR, ^1H NMR and ^{13}C NMR spectra, but opposite optical rotation as 17β-estradiol.

A. Preparation of (8α,9β,10α,13α,14β,17α)-17-hydroxyestr-4-en-3-one (*ent*-19-nortestosterone).

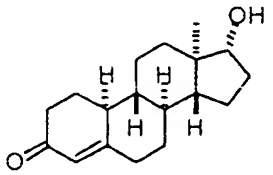
Method A.

Ammonia gas was condensed in a 500 ml three-necked round bottom flask cooled to −78 °C (cooling bath: dry ice, 2-propanol) under nitrogen until 200 ml of liquid ammonia was collected. Freshly cut lithium (1.4 g, 200 mmol) was added and the reaction solution was stirrred (overhead mechanical stirrer) for 15 minutes. To the blue colored reaction solution, [3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one (4g, 10 mmol) in dry tetrahydrofuran (THF; 100 ml) was added and the reaction solution was stirred for 1 hour. The blue color persisted during this time. After 1 hour, solid ammonium chloride (5g) was added slowly and carefully while maintaining the temperature at −78 °C. The blue colored solution turned into a milky white solution on addition of the ammonium chloride. The cooling bath was removed and the reaction mixture was then left overnight during which time the liquid ammonia became gaseous ammonia and evaporated. Water (200 ml) was added and the reaction mixture was extracted with ethyl acetate (3 × 100 ml). The combined ethyl acetate extracts were washed with brine (100 ml). The solvents were removed and the residue obtained was chromatographed on a silica gel column eluted with 20% ethyl acetate in hexanes to give pure [3*R*-(3α,3aα,5aβ,6β,9aα,9bβ)]-3-(1,1-dimethylethoxy)-dodecahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one (3g, 75%). A portion of this material was then converted into *ent*-19-nortestosterone as described below. To the above [3*R*-(3α,3aα,5aβ,6β,9aα,9bβ)]-3-(1,1-dimethylethoxy)-dodecahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one (2g, 5.12 mmol) in methanol (100 ml), 3N hydrochloric acid (30 ml) was added and the reaction solution was refluxed for 24 hours. The reaction solution was then poured into water and the aqueous reaction mixture was extracted with ethyl acetate (2× 100 ml). The combined extracts were washed with brine. The crude product was chromatographed on a silica gel column eluted with 20% ethyl acetate in hexanes mixture to give pure *ent*-19-nortestosterone (0.5g. 57 %) as a white crystalline solid which had physical properties identical to those reported below when this compound was prepared using Method B.

Method B.

[3*R*-(3α,3aα,9aα,9bβ)]-3-(1,1-Dimethylethoxy)-1,2,3,3a,4,5,8,9,9a,9b-decahydro-3a-methyl-6-[2-(2-methyl-1,3-dioxolan-2-yl)ethyl-7*H*-benz[*e*]inden-7-one, 18.35 g, 47 mmol) was dissolved in ethanol (EtOH; 180 ml) in a Parr hydrogenation bottle used with a Parr hydrogenation apparatus. Pd/C catalyst (1.00g, 9.4 mmol) was added, air was removed, the reaction vessel was pressurized to 60 psi with hydrogen gas and the rocker motor was started. The hydrogenation reaction was carried out for 4 hours. At the conclusion of this time, the reaction mixture was filtered through a bed of Celite. Solvent removal gave an oil which was used immediately in the next step.

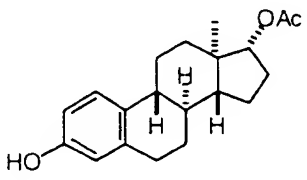
The oil was dissolved in EtOH (50 ml) and 6N HCl was added (50 ml). The reaction solution was then refluxed. After 48 hours, the reaction mixture was neutralized by the addition of solid $NaHCO_3$ until the pH was 8–9. Volatile solvents were removed and the aqueous residue was extracted with methylene chloride (3 portions of 200 ml each). The organic extracts were combined, dried with $MgSO_4$, filtered, and the solvents were removed to afford an oil (12.5 g). ^{1}H NMR analysis revealed a complex mixture of products. Purification by colum chromatography (silica gel eluted with 19-37% ethyl acetate in hexanes), followed by recrystallization gave *ent*-19-nortestosterone (2.04 g, 7.45 mmol, 16% yield): [α] = −57.3° (c = 0.99,$CHCl_3$); mp 124-125 °C; IR 3425, 2926, 2866, 1661, 1619, 1450, 1334, 1261, 1208, 1135, 1056 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 5.81 (1 H, s), 3.65 (1 H, t, *J* = 8.4 Hz), 2.5-2.0 (7 H, m), 1.90-1.75 (3 H, m), 1.70-0.80 (11 H, m), 0.79 (3 H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.05, 166.72, 124.64, 81.66, 49.70, 49.53, 42.93, 42.52, 40.43, 36.39, 36.33, 35.37, 30.59, 30.32, 26.48, 26.02, 23.07, 10.89; Anal. Calcd for $C_{18}H_{26}O_2$: C, 78.79; H, 9.55; Found: C, 79.04, H, 9.41.



B. Preparation of (8α,9β,13α,14β,17α)-estra-1,3,5(10)-triene-3,17-diol,17acetate (*ent*-17β-estradiol,17-acetate).

Acetic anhydride (1.85 ml) was mixed with pyridine (5 ml) and stirred under nitrogen for 45 minutes and the *ent*-17β-nontestosterone (304 mg, 1.11 mmol) was added.

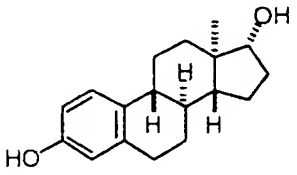
The reaction vessel was purged with nitrogen and the reaction solution was stirred overnight. The following morning, 0.5 M HCl (15 ml) was added. After stirring for 1 hour, the reaction mixture was extracted with methylene chloride (3 portions of 30 ml). The combined organic extracts were washed with 1 N HCl (2 portions of 30 ml), saturated $NaHCO_3$ (1 portion of 30 ml) and brine (1 portion of 30 ml). The combined extracts were then dried with $MgSO_4$, filtered, and the solvents removed to give a yellow oil (0.37 g). Purification by chromatography (silica gel eluted with 20% ethyl acetate in hexanes) gave *ent*-19-nortestosterone,17-acetate as a colorless oil (0.32 g, 1.01 mmol, 91% yield) which was used in the next reaction. *Ent*-19-nortestosterone,17-acetate (0.32 g, 1.01 mmol) was dissolved in acetonitrile (10 ml). $CuBr_2$ (0.28 g, 1.25 mmol, 1.24 equivalents) was added. The reaction vessel was purged with nitrogen and the reaction was stirred overnight. The following morning, additional $CuBr_2$ (0.14 g, 0.63 mmol, 0.62 equivalents) was added. After an additional 2 hours, the reaction was quenched by the addition of water (15 ml). The acetonitrile was removed under reduced pressure. Additional water (10 ml) and brine (10 ml) were added. The reaction mixture was extracted with ethyl acetate (3 40 ml). The combined organic extracts were washed with brine (2 40 ml), dried with $MgSO_4$, filtered, and the solvents were removed to give a yellow solid (0.35 g). Purification by chromatography (silica gel, eluted with 20% ethyl acetate in hexanes) gave *ent*-17β-estradiol, 17-acetate (0.24 g, 0.76 mmol, 76% yield) as a white solid: mp 219-21°C; IR 3419, 2927, 2871, 1708, 1611, 1585, 1546, 1500, 1447, 1375, 1358, 1274, 1181, 1153, 1132, 1039, 962 cm^{-1}; ^{1}HNMR (300 MHz, $CDCl_3$) δ 7.14 (1 H, d, J = 8.4 Hz), 6.64-6.56 (2 H, m), 5.00 (1 H, s), 4.69 (1 H, dd, J = 7.8 Hz, 9.3 Hz), 2.82 (2 H, m), 2.06 (3 H, s), 0.82 (3 H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.59, 153.58, 138.27, 132.61, 126.60, 115.32, 112.77, 82.84, 49.73, 43.72, 42.85, 38.50, 36.83, 29.48, 27.47, 27.07, 26.12, 23.14, 21.08, 11.93.



C. Preparation of (8α,9β,13α,14β,17α)-estra-1,3,5(10)-triene-3,17-diol (*Ent*-17β-estradiol).

The compound of (B), above, (0.21 g, 0.668 mmol) was dissolved in stirred EtOH (25 ml) and 10% aqueous NaOH (2.5 ml) was added. The reaction vessel was purged with nitrogen and the reaction was allowed to proceed overnight. The following morning, the reaction solution was quenched by the addition of 1N HCl (2 ml) and brine (50 ml). The reaction mixture was extracted with ethyl acetate (3 portions of 60 ml). The combined organic extracts were washed with brine (2 portions of 50 ml), filtered, and the solvents were removed to give a yellowish solid (0.20 g). Purification by chromatography gave

ent-17β-estradiol as a white solid (174 mg, 0.64 mmol, 96%): mp 176-177 °C; [α] = −71.2 (c = 0.99, CH_3OH); IR 3449, 3246, 2936, 2864, 1611, 1587, 1500, 1450, 1283, 1250, 1057, 1012, 930, 874 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 7.06 (1 H, d, *J* = 8.7 Hz), 6.54-6.46 (2 H, m), 3.64 (1 H, t, *J* = 8.4 Hz), 0.75 (3 H); ^{13}C NMR (75 Hz,CD_3OD) δ 156.07, 138.98, 132.80, 127.32, 116.18, 113.85, 82.57, 51.32, 45.34, 44.36, 40.50, 38.01, 30.67 (2 C), 28.48, 27.56, 23.99, 11.62.



Example 7. Treatment of Stroke with *Ent*-17β-Estradiol.

Sprague-Dawley female rats (20-225 grams body weight) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). They were housed in pairs in hanging, stainless steel cage in a temperature controlled room (25°C) with daily light cycle (light on 0700 to 1900 h daily) for a minimum of 3 days before surgery. All rats had free access to Purina Rat Chow and tap water. All procedures performed on animals were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida before initiation of the study. Animals were ovariectomized at 1 week prior to middle cerebral artery (MCA) occlusion. At 2 hours prior to the MCA occlusion, animals received a subcutaneous injection of one of the following: corn oil vehicle (1 mg/kg body weight), 17β-estradiol (100 μg/kg body weight) or *ent*-17β-estradiol (100 μg/kg body weight). MCAO was achieved according to the methods

described previously by us. Briefly, following administration of anesthetics of ketamine (60 mg/kg, ip) and xylazine (10 mg/kg, ip), the common carotid artery (ICA) on the left side were exposed through a midline cervical incision and then gently dissected away from adjacent nerves. A 3-O monofilament nylon suture was introduced into the left MCA lumen and gently advanced to the distal ICA until resistance was felt where the suture passed the bifurcation of the MCA and anterior cerebral artery (ACA). The thread was left in place for 60 minutes after which time re-perfusion was initiated. Rectal temperature was monitored and maintained between 36.5 and 37.0°C during the entire stroke procedure. Each group of animals was decapitated after 24 hours of reperfusion. The brain was removed and placed in a metallic brain matrix for tissue slicing immediately after sacrifice. Five slices were made at 3, 4, 7, 9 and 11 m posterior to the olfactory bulb. The slices were incubated for 30 minutes in a 2% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) in physiological saline at 37°C and then were fixed in 10% formalin. The stained slices were photographed and subsequently measured for the surface area of the slices and the ischemic lesion area. Ischemic lesion volume was calculated as by the sums of the areas of ischemic lesion across the five slices divided by the total cross sectional area of these five brain slices.

The results of pretreatment with 17β-estradiol or *ent*-17β-estradiol on the mean area for vehicle controls was 13.3 ±2.0 (mean ± SEM). Treatment with 17β-estradiol reduced the infarct to 5.3 ± 1.6, a reduction of 60%. Treatment with *ent*-17β-estradiol caused a similar decline in infarct area to 5.3 ± 1.7. Dunn's Multiple Comparison test revealed that both the 17β-estradiol and the *ent*-17β-estradiol were significantly different from the vehicle control ($p<0.05$) but not from each other.

Plasma 17β-estradiol levels following *ent*-E2 administration did not change from the pre-injection baseline of 0.05 ± 0.01 nM (Fig. 6). In contrast, subcutaneous injection of 17β-estradiol resulted in a rapid rise in plasma 17β-estradiol levels with values of 5.16 ± 0.94 nM within 1 h and returned to near baseline (0.24 ± 0.08) by 24 h.

Example 8

Neuroprotective effects of the enantiomer of 17β-estradiol (ent-E2) both *in vitro* and *in vivo* can be disassociated from peripheral estrogenic activities. Ent-E2 provides neuroprotection without the sex related activity of β-estradiol and therefore can be used to treat men or those women who are predicted to react adversely to β-estradiol. This

example shows that ent-E2 can exert neuroprotective effects in the absence of stimulation of female reproduction associated effects. Ent-E2 was made according to Example 6. 17β-estradiol was purchased from Steraloids, Inc. (Wilton, NH).

Steroids were initially dissolved in ethanol at a 10 mM concentration and then diluted to the appropriate concentration in culture media or assay buffer for cell culture or *ex vivo* assays, respectively. Steroids were dissolved in corn oil at the concentration necessary to yield the indicated dose in 1 ml/kg injection volume for rodent studies. Rats were bred as described in Example 7.

Cell Culture

SK-N-SH human neuroblastoma cells were obtained from ATCC (Rockville, MD) and HT-22 cells (immortalized hippocampal neurons of murine origin) were obtained from the Salk Institute, San Diego, CA. Cells were maintained in RMPI-1640 and DMEM media (GIBCO, Gaithersburg, MD), respectively, supplemented with 10% charcoal/dextran-stripped fetal bovine serum (Hyclone, Logan, UT) and 200 µg/ml gentamycin according to standard culture conditions.

Cells were plated 24 hours prior to initiation of experiment at a density of $2x10^4$ cells/well (SK-N-SH cells) or $5x10^3$ cells/well (HT-22 cells) in Nunc® 96-well plates (Fisher Scientific, Orlando, FL). Steroids were added at a concentrations ranging from 0.1 nM to 10 µM either 2 or 24 hours prior to exposure to either glutamate (5 mM) or H_2O_2 (3-60 µM). Ethanol was used at a concentrations of 0.001 to 0.1% v/v as a vehicle control. These concentrations of ethanol had no discernable effect on cell viability. Following 24 hours of toxin expsoure, cells were rinsed with PBS, pH 7.4 and viability was assessed by the addition of 1 µM calcein AM (Molecular Probes, Eugene, OR) and 1 µg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) in PBS for 15 min. Calcein AM fluorescence was determined at an excitation of 485 nm and an emission of 538 nm. Cells which had been lysed by addition of 1% SDS was used for blank readings. Staining was visualized using a fluorescent Nikon microscope and cells were photographed for qualitative documentation.

Ovariectomy

Female Sprague-Dawley rats (220-225 g body weight) were given 3-5 days to acclimate then were bilaterally ovariectomized using a dorsal approach. Animals were anesthetized with methoxyflurane (Pitman Moore, Inc., Crossing, NJ) inhalant anesthesia.

A small (1 cm) cut was made through the skin, facia, and muscle. The ovaries were externalized, clipped, and removed then the muscle, facia, and skin were sutured closed. Ovariectomy was performed 2 weeks prior to experiments.

***Plasma levels of* 17β-estradiol**

Ovariectomized female Sprague-Dawley rats were injected subcutaneously with either oil vehicle or 100 μg/ml of 17β-estradiol or *ent*-E2. Blood samples were obtained by cardiac puncture 5 min prior to injection or 1 h, 4 h, or 24 h post-injection. Plasma was stored at

-20°C until assayed using the ultra-sensitive 17β-estradiol RIA kit from Diagnostic Systems Laboratories, Inc (Los Angeles, CA) according to the manufacturer's instructions. *Ent*-E2 showed no cross-reactivity with the RIA at concentrations up to 10 μM.

Uterotrophic Assay

Juvenile (25 day old) female Sprague-Dawley rats were injected subcutaneously with oil, 17β-estradiol (0.01 to 1 μg/rat), or *ent*-E2 (1 to 100 μg/rat) daily (0830) for 3 days. On the fourth day, the rats were euthanized using methoxyflurane and the uteri excised. Extraneous tissue was gently removed from the uteri before wet weight was determined. Vaginal opening was assessed prior to uterine removal.

Ligand Competition of Estrogen Receptor Binding

5 nM [2,4,6,7-^{3}H]-17β-estradiol (specific activity 84.1 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ) and 400 pM recombinant human estrogen receptor (ER) α or β (Affinity Bioreagents, Inc., Golden, CO) in ER binding buffer (20 mM Tris, 1 mM EDTA, 400 mM KCl, 1 mM DTT, 10 % glycerol, 0.1 % BSA, pH 7.8) was incubated for 1 hour at 25°C with no added steroid (total binding), 1.2 μM diethylstilbesterol (non-specific binding), or 0.1 nM to 10 μM 17β-estradiol or *ent*-E2. Bound and unbound radioligand was separated using Sephadex G-25 (Amersham Pharmacia Biotech) columns (1.5 ml bed volume) with a 1 ml elution volume. 10 ml scintillation fluid was added and counts determined. This method resulted in greater than 90 % receptor recovery and less than 15 % non-specific binding.

Brain Membrane Oxidation

The brain was removed from ovariectomized female Sprague-Dawley rat and the neocortex was homogenized in ice-cold Tris buffer (100 mM, pH 7.4) with 1 % Triton X-

100 using a Teflon/glass tissue homogenizer. The homogenate was centrifuged at 2000 rpm for 10 min. The resulting supernatant was incubated with 17β-estradiol or *ent*-E2 at concentrations ranging from 0.1 to 100 μM for 30 min at 37°C. $FeSO_4$ was then added to a final concentration of 200 μM and incubated for an addtional 30 min at 37°C. BHT (100 μM) and DPTA (100 μM) were then added. 2-thiobarbituric acid reactive products (TBARs) were immediately determined by addition of 0.5% 2-thiobarbituric acid, 3.125% tricholoroacetic acid and 0.2 N HCl and incubation at 95°C for 1 hour. Samples were centrifuged at 10000 rpm for 10 min and the absorbance of the supernatant at 532 nm determined.

Statistical Analysis

All data are presented as mean ± sem. Comparison of ischemic lesion volume was performed using a one-way ANOVA with a Kruskal-Wallis test for planned comparisons between groups. For all other experiments, the significance of differences among groups was determined by one-way ANOVA with a Tukey's Multiple Comparisons Test for planned comparisons between groups when a significant difference was detected. For all tests, $p<0.05$ was considered significant.

Results

Ent-E2 attenuates oxidative stress-induced death in neuronal cultures.

HT-22 cells, transformed hippocampal neurons, are sensitive to glutamate toxicity via a mechanism which involves glutathione depletion and the resulting oxidative stress (31). Exposure of HT-22 cells to 10 mM glutamate caused a 70 - 75 % reduction in neuronal viability by 24 h of exposure (Fig. 2). Exposure to 17β-estradiol conferred significant protection in this model with a 10 μM concentration protecting 35 ± 4% of the cells. *Ent*-E2 performed similarly in this model of neuroprotection with 0.1 μM and 10 μM of *ent*-E2 protecting 16 ± 2 % and 56 ± 4 % of HT-22 cells, respectively.

In another model of oxidative stress, both 17β-estradiol and *ent*-E2 significantly attenuated H_2O_2-induced toxicity in HT-22 cells (Fig. 3). H_2O_2 exposure resulted in a concentration-dependent toxicity in HT-22 cells with a 30 μM concentration resulting in 21 ± 5 % reduction in viability and a 60 μM concentration resulting in a 97 ± 8 % reduction. 10 nM of either 17β-estradiol or *ent*-E2 completely attenuated the toxicity of

30 μM H_2O_2 and protected 48 ± 14 % and 63 ± 8 % of the cells from 40 μM H_2O_2 toxicity, respectively (Fig. 3). No protection was seen with the 10 nM concentration of either steroid at H_2O_2 concentrations greater than 40 μM (data not shown). SK-N-SH cells were more sensitive than HT-22 cells to the toxic effects of H_2O_2 exposure with 3 μM H_2O_2 reducing SK-N-SH cell viability by 32 ± 2 %. Cell death was almost completely attenuated by 10 nM *ent*-E2 with a 1 nM concentration conferring 30 ± 9 % protection (Fig. 4). This is comparable to neuroprotection observed with 1 nM 17β-estradiol which we found to prevent 40 ± 5 % of H_2O_2-induced toxicity in SK-N-SH cells.

Ent-E2 is a weak ER agonist/antagonist.

Daily administration of 17β-estradiol for 3 days caused a dose-dependent increase in uterine wet weight with a 1 μg/rat dose (average dose of 13.8 μg/kg) increasing wet uterine weight by two-fold (Fig. 7). By contrast, *ent*-E2 at doses of 1 to 10 μg/rat had no effect on uterine wet weight. At a dose of 100 μg/rat (average dose of 1400 μg/kg), *ent*-E2 exerted a slight anti-uterotrophic effect, decreasing uterine wet weight by 23 ± 3 %. *Ent*-E2 also slightly antagonized the utertrophic effects of 1 μg/rat 17β-estradiol with a 100 μg/rat dose reducing the uterotrophic effect of 17β-estradiol by 27 ± 8 %. These results are comparable to previous reports in immature mice where *ent*-E2 (doses of about 1200 μg/kg) exerted anti-uterotrophic effects (20) and *ent*-E2 antagonized the uterotrophic effects of 17β-estradiol when *ent*-E2 was present in a 100-fold excess (23).

Daily injections of 17β-estradiol (1 μg/rat) induced vaginal opening in 100 % of the animals examined (Table 1). Ent-E2 exerted mixed agonist/antagonist effects on vaginal opening with a 100 μg/rat dose causing vaginal opening in 50 % of the juvenile rats. This dose of *ent*-E2 prevented 17β-estradiol-induced vaginal opening in 40 % of the rats. No change in body weight was observed with administration 17β-estradiol, *ent*-E2, or combinations thereof. Body weights of the juvenile rats averaged 72 ± 1g.

In competition binding experiments, *Ent*-E2 showed weak binding to both known estrogen receptors with 4.2 % and 6.3 % of the relative binding affinity of 17β-estradiol to ERα and ERβ, respectively.

Ent-E2 can attenuate brain lipid oxidation ex vivo.

We examined the potency of both 17β-estradiol and *ent*-E2 in an *ex vivo* assay of brain membrane oxidation. 30 min incubation of the neocortical homogenate resulted in a

16-fold increase in TBAR formation. 17β-estradiol and *ent*-E2 were equipotent in the attenuation of FeSO4-induced lipid oxidation as determined by TBAR formation (Fig. 8) with a 50 μM concentration of either steroid significantly attenuating $FeSO_4$-induced TBAR formation.

In summary, *ent*-E2 was both as potent and efficacious as 17β-estradiol in culture models of neuroprotection and further, *ent*-E2 reduced ischemic lesion volume following MCA occlusion to the same degree as 17β-estradiol. In contrast, *ent*-E2 showed only minimal binding affinity for either known estrogen receptor, was at least 100-fold less potent than 17β-estradiol in exerting effects on uterine growth or vaginal opening, and had weak anti-uterotrophic effects. These data indicate that the neuroprotective effects of estrogens can occur without stimulation of peripheral estrogen-responsive tissues.

The neuroprotective effects of *ent*-E2 are not likely due to conversion to the more potent 17β-estradiol as the conversion requires isomerization of five individual chiral carbons. Isomerization of the 17-hydroxy group could be facilitated by 17β-hydroxy steroid dehydrogenase; however, *ent*-E2 is not a substrate for this enzyme (Segal GM, Cherkasov AN, Torgov IV 1967 Enzymatic transformation of the dl-estradiol into d-estrone and l-estradiol. Khim Prir Soedin 3:304-307). Further, there was no detectable increase in plasma 17β-estradiol levels during 24 hours following subcutaneous injection of *ent*-E2 in female rats indicating that *ent*-E2 is itself neuroprotective.

Table 1. Effects of Pretreatment with 17β-estradiol or an Estradiol Chemical Delivery System (E2-CDS) on Mortality Following Middle Cerebral Artery Occlusion.

Treatment	Time of Planned Sacrifice	Number of Animals Tested	Number of Animals Alive	Number of Animals Dead	% Survival
Sham	6 hrs	12	5	7	42
	1 day	18	6	12	33
	1 week	5	1	4	20
	Total	35	12	23	35
E2 Implant	6 hrs	6	3	3	50
	1 day	8	8	0	100*
	1 week	4	3	1	75*
	Total	18	14	4	78*
E2-CDS	6 hrs	7	5	2	71
	1 day	8	7	1	88*
	1 week	4	4	0	100
	Total	19	16	3	84*

*$p<0.05$ versus sham control group at each of the time points, as determined by Chi Squares analysis.

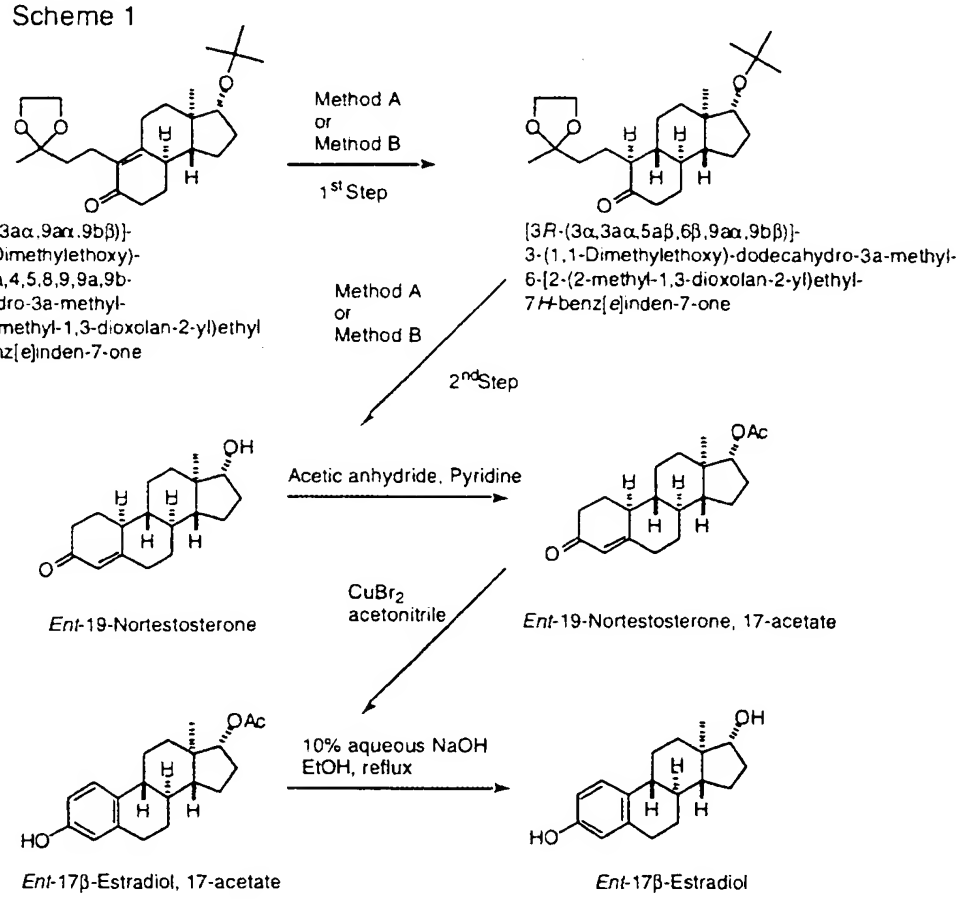
Table 2. Effects of Estrogens on the Area Under the Ischemic Lesion Curve in Ovariectomized Rats.

Steroid	Treatment	Area Under Curve
Sham	24 hour pretreatment	8.1±0.8
17α-estradiol	24 hour pretreatment	3.7 ±1.3*
HPCD Vehicle	40 min post-treatment	10.1 ±1.6
E2-CDS	40 min post-treatment	4.5 ±0.9*
HPCD Vehicle	90 min post-treatment	8.2 ±1.7
E2-CDS	90 min post-treatment	5.21±1.7

* $p<0.02$ versus sham control by Students t test

Table 3: Diagram of the Synthesis of *Ent*-17-β-estradiol.

Scheme 1



Reagents: Method A, 1st Step, Li, liquid NH_3, THF; 2nd Step, 3N HCl, EtOH, reflux
Method B, 1st Step, H_2, Pd/C, EtOH; 2nd Step, 6N HCl, EtOH, reflux

Table 4. Effects of 17α-estradiol and *Ent*-E2 on Vaginal Opening in Juvenile Female Rats

Ent-E2 Dose (μg/rat)	Number with Vaginal Opening	
	Without 17α-estradiol	With 1μg/rat 17α-estradiol
0	0 of 4	5 of 5
10	1 of 4	3 of 4
100	2 of 4	3 of 5

25 day old female Sprague-Dawley rats were injected subcutaneously with the indicated dose of *Ent*-E2 with or with concurrent administration of 1 μg/kg 17α-estradiol daily for 3 days. On day 4, vaginal opening was assessed.

We claim:

1. A method for conferring protection on a population of cells associated with ischemia in a subject, comprising:

a) providing an enantiomer of an estrogen compound; and

b) administering an effective amount of the compound over a course that includes at least one dose within a time that is effectively proximate to the ischemic event, so as to confer protection on the population of cells.

2. A method according to claim 1, wherein the proximate time precedes the ischemic event.

3. A method according to claim 1, wherein the proximate time follows the ischemic event.

4. A method according to claim 1, wherein the proximate time is within 12 hours of the ischemic event.

5. A method according to claim 1, wherein the ischemic event is selected from the group consisting of a cerebrovascular disease, stroke, subarachnoid hemorrhage, myocardial infarct, surgery and trauma.

6. A method according to claim 1, wherein the ischemic event is a stroke.

7. A method according to claim 1, wherein the ischemic event is a myocardial infarct.

8. A method according to claim 6, wherein the cells are neurons.

9. A method according to claim 8, wherein the cells are endothelial cells.

10. A method according to claim 8, wherein the cells are cardiac myocytes.

11. A method according to claim 1, wherein the enantiomer of the estrogen compound is administered at an effective dose, wherein the effective dose provides a plasma concentration in the subject in the range of 10-500 pg/ml.

12. A method according to claim 1, wherein the estrogen compound *is Ent*-17β-estradiol.

13. A method for conferring protection on a population of cells associated with ischemia, in a subject following an ischemic event, comprising:

a) providing an enantiomer of an estrogen compound formulated in an oil vehicle; and

b) administering an effective amount of the compound over a course that includes at least one dose within a time that is effectively proximate to the ischemic event, so as to confer protection on the population of cells.

14. A method for treating a neurodegenerative disorder in a subject, comprising: providing an enantiomer of an estrogen compound in a pharmaceutical formulation; and administering the formulation to the subject.

15. A method according to claim 13 or 14, wherein the formulation is administered by a route selected from the group consisting of subcutaneous, transdermal and intravenous.

16. A method according to claim 13 or 14, wherein step (b) further comprises; administering the estrogen compound by subcutaneous injection.

17. A method according to claim 13 or 14, wherein step (b) further comprises; administering the enantiomer of the estrogen compound intravenously.

18. A method according to any of claims 13 through 17, wherein the enantiomer is *Ent*-17β-estradiol.

19. A composition, comprising *Ent*-17β-estradiol,17-acetate.

20. A method for conferring a cytoprotective effect on a population of cells in a male or female subject, comprising:

(A) providing an estrogen compound or an enantiomer of an estrogen compound having insubstantial sex related activity, in a pharmaceutical formulation; and

(B) administering the formulation in an effective dose to the population of cells to confer cytoprotection.

21. A method according to claim 20, wherein step (a) further comprises providing an effective dose of the estrogen compound or an enantiomer of an estrogen compound in a pharmaceutical formulation and step (b) further comprises administering the formulation to a subject so as to retard the adverse effects of a degenerative condition.

22. A method according to claim 21, wherein the degenerative condition is selected from an acute degenerative condition and a chronic degenerative condition.

23. A method according to claim 22, wherein the acute degenerative condition includes ischemia.

24. A method according to claim 22, wherein the chronic degenerative condition includes a neurodegenerative diseases exemplified by Alzheimer's disease or an osteo-degenerative diseases exemplified by osteoporosis.

24. A pharmaceutical formulation, comprising: an entiomer of an estrogen compound in an oil containing formulation.

25. A method of administering an estrogen compound or enantiomer of an estrogen compound , comprising:

selecting an effective dose of the estrogen compound or enantiomer; and

administering the effective dose subcutaneously.

5

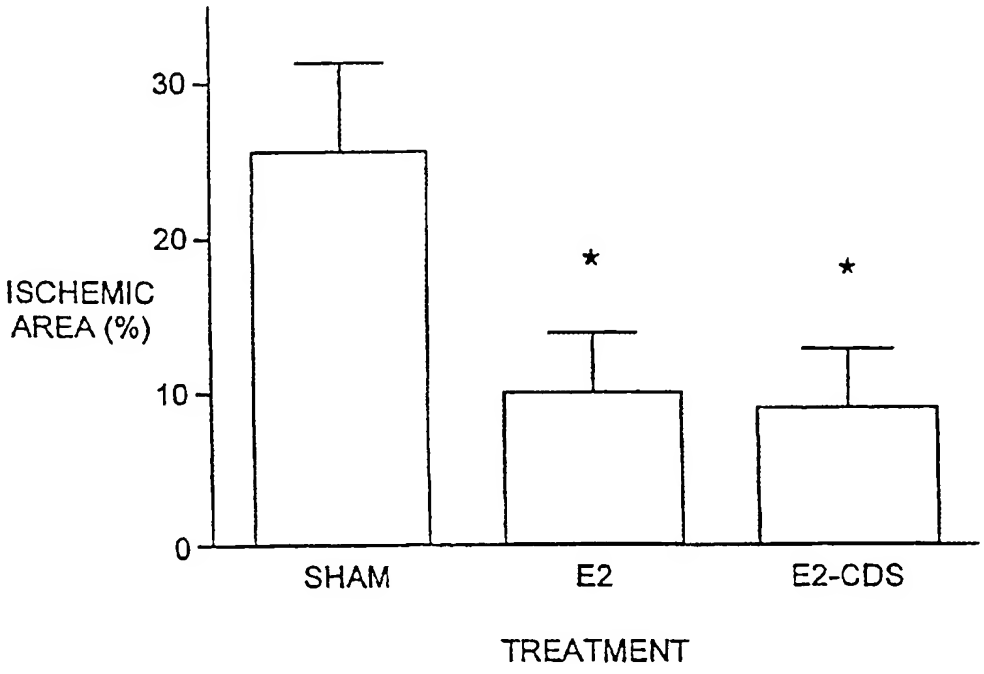


FIG. 1

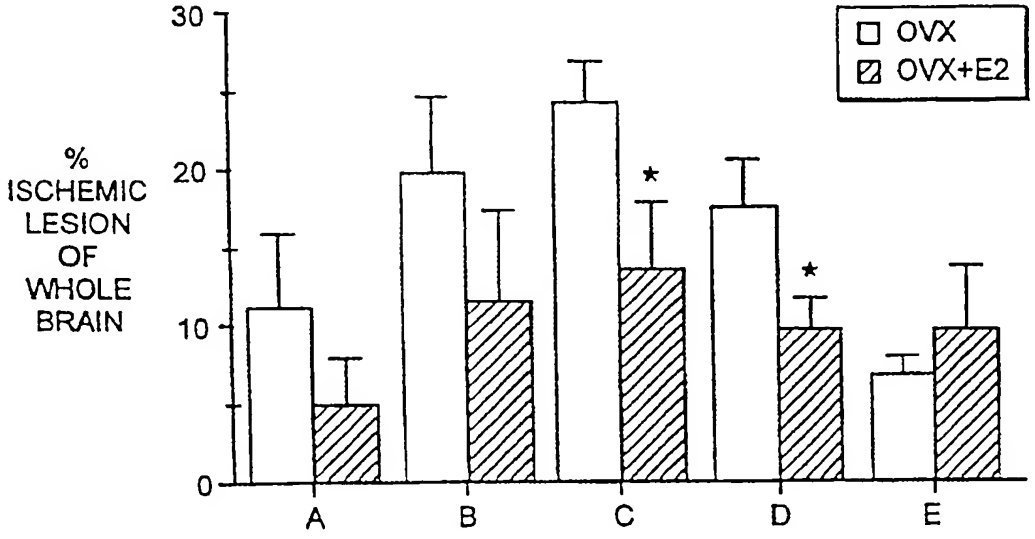


FIG. 2

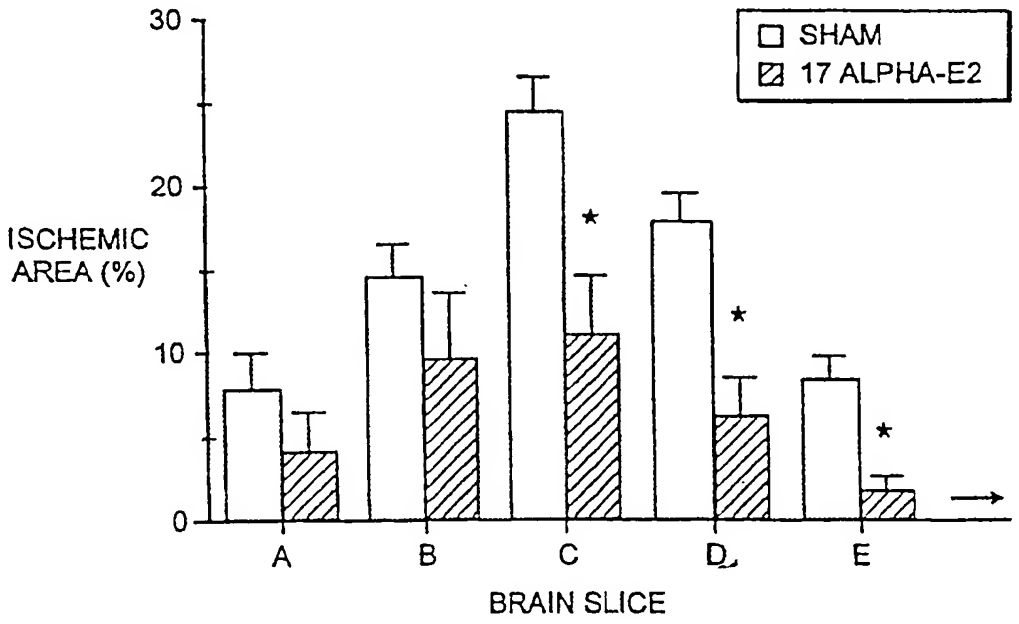


FIG. 3

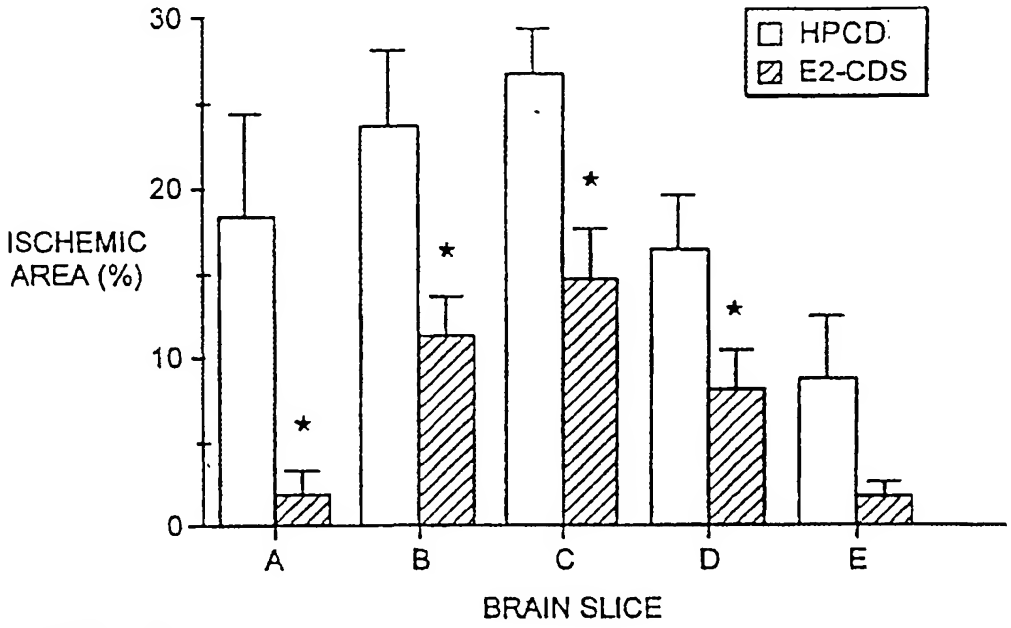

30
20
10
0
ISCHEMIC AREA (%)
HPCD
E2-CDS
*
*
*
*
A
B
C
D
E
BRAIN SLICE
40 MINS

FIG. 4a

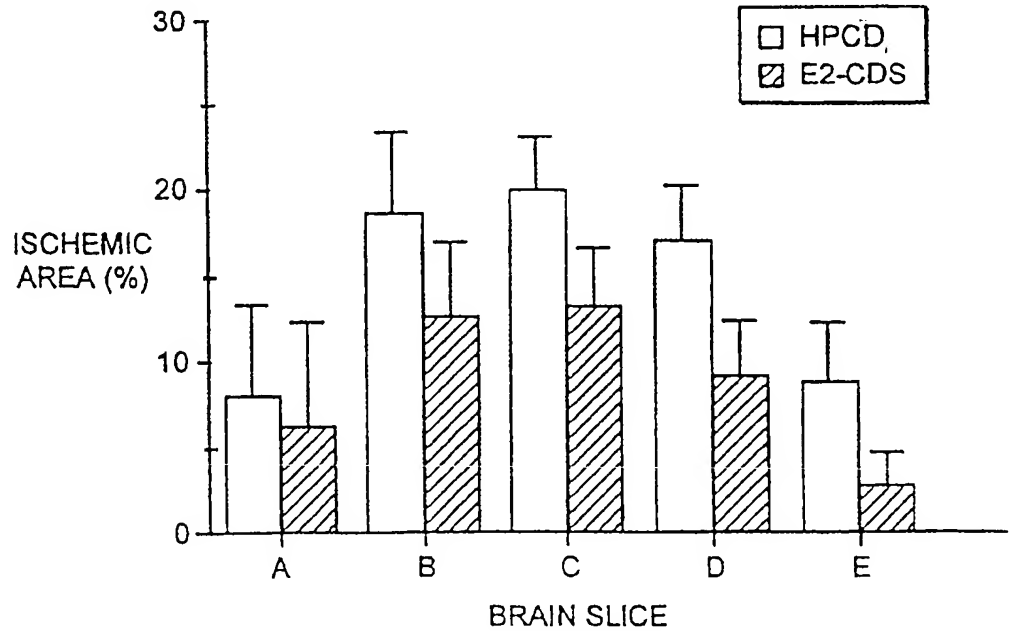

30
20
10
0
ISCHEMIC AREA (%)
HPCD
E2-CDS
A
B
C
D
E
BRAIN SLICE
90 MINS

FIG. 4b

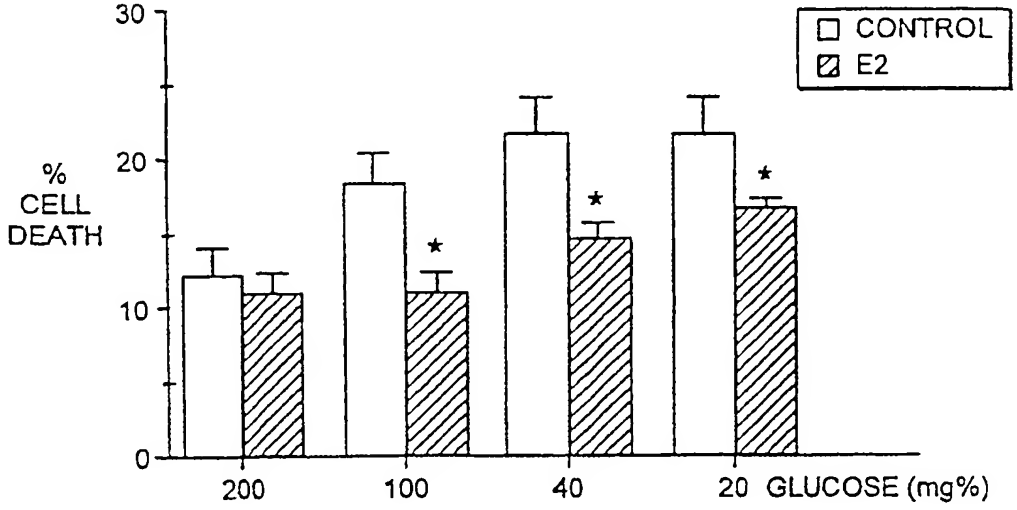

30
20
10
0
% CELL DEATH
CONTROL
E2
*
*
*
200
100
40
20
GLUCOSE (mg%)

FIG. 5a

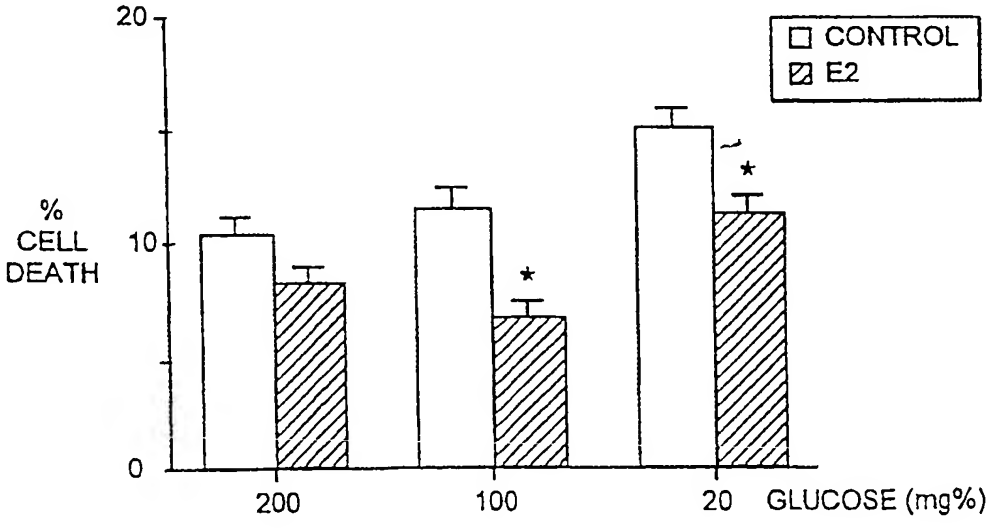

20
10
0
% CELL DEATH
CONTROL
E2
*
*
200
100
20
GLUCOSE (mg%)

FIG. 5b

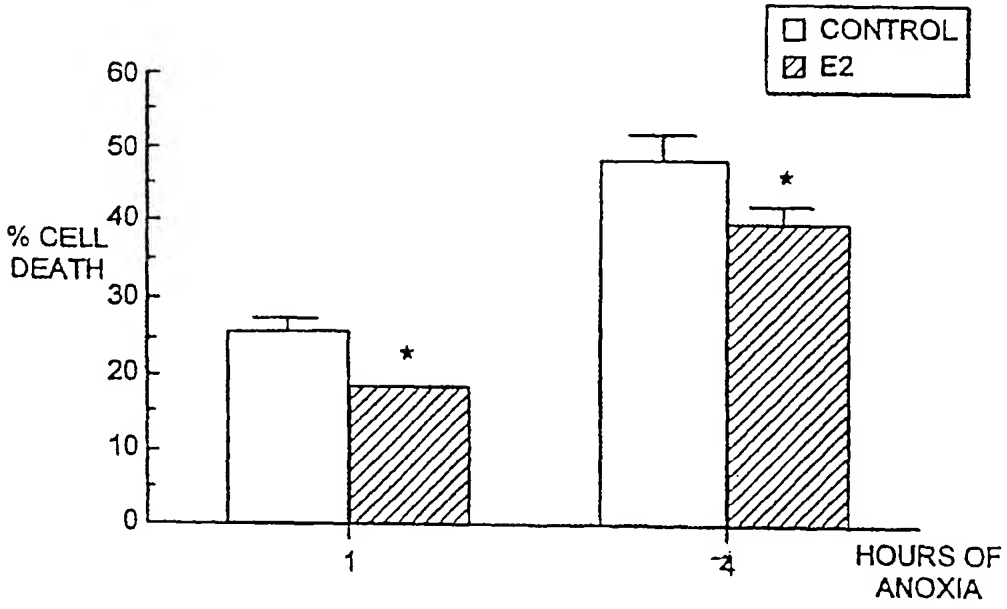


FIG. 6


60
50
40
30
20
10
0
%CELL DEATH
CONTROL
E2
*
*
*
+O2
-O2
+O2
-O2
200 mg% GLUCOSE
100 mg% GLUCOSE

FIG. 7

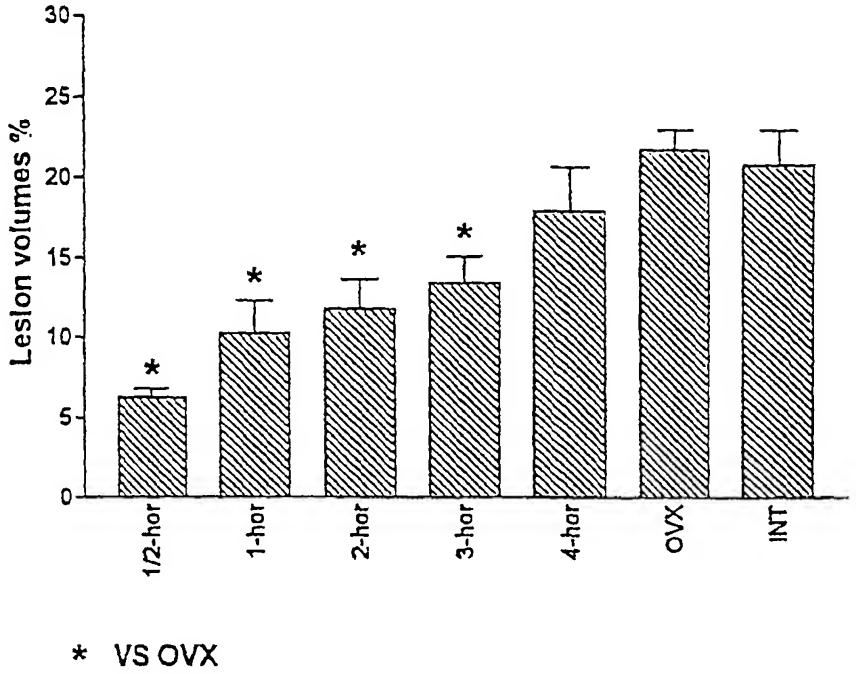

Lesion volumes %
30
25
20
15
10
5
0
1/2-hor
1-hor
2-hor
3-hor
4-hor
OVX
INT
*
* VS OVX

FIG. 8

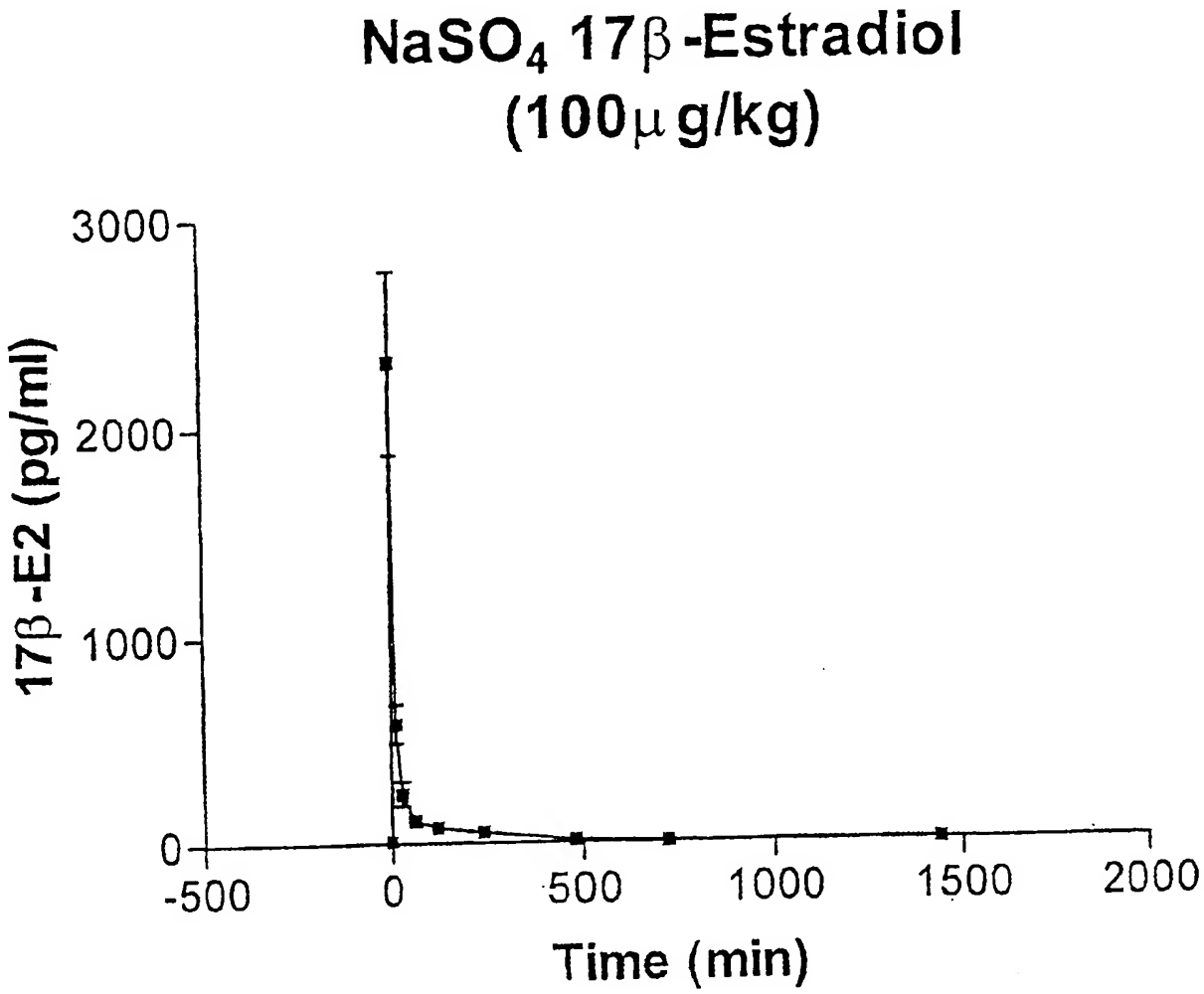
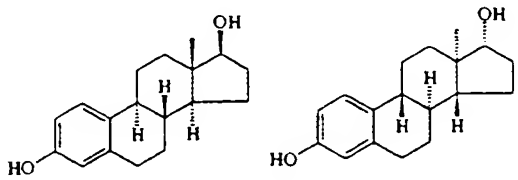

NaSO4 17β-Estradiol
(100μg/kg)
3000
2000
1000
0
17β-E2 (pg/ml)
-500
0
500
1000
1500
2000
Time (min)

FIG. 9



17β-estradiol Ent-estradiol

FIG. 10

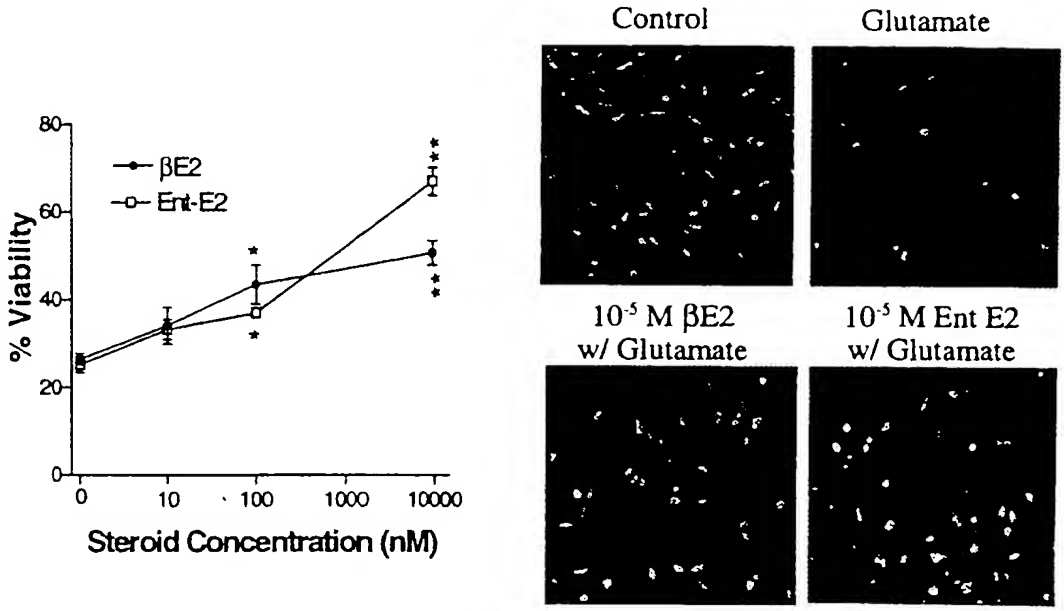

Control
Glutamate
βE2
Ent-E2
% Viability
80
60
40
20
0
0
10
100
1000
10000
Steroid Concentration (nM)
10^{-5} M βE2
w/ Glutamate
10^{-5} M Ent E2
w/ Glutamate

FIG. 11

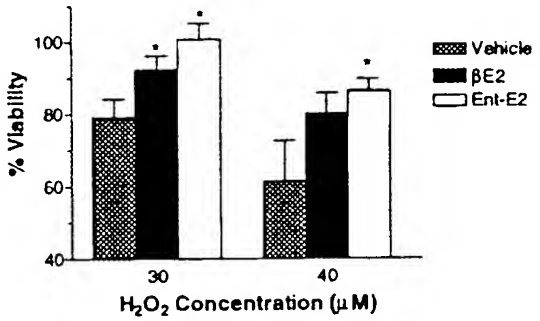

% Viability
100
80
60
40
30
40
H_2O_2 Concentration (μM)
Vehicle
βE2
Ent-E2

FIG. 12

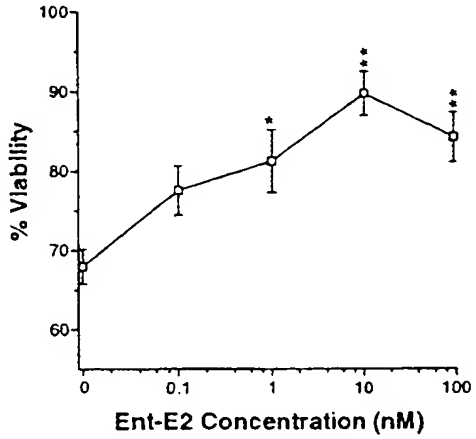


FIG. 13

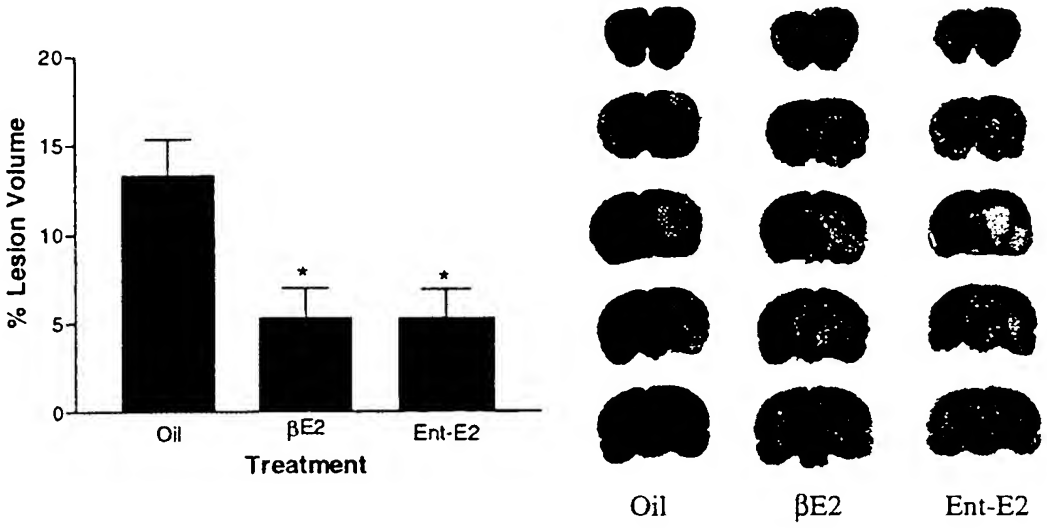

% Lesion Volume
20
15
10
5
0
Oil
βE2
Ent-E2
Treatment
Oil
βE2
Ent-E2

FIG. 14

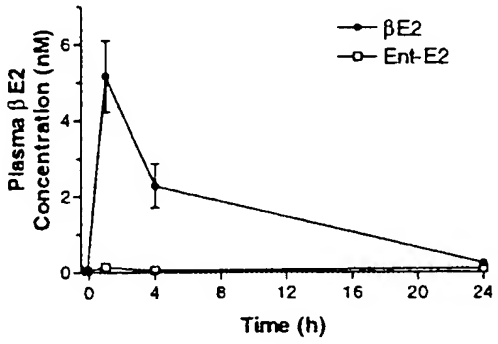

βE2
Ent-E2
Plasma β E2 Concentration (nM)
0
2
4
6
0
4
8
12
16
20
24
Time (h)

Fig. 15

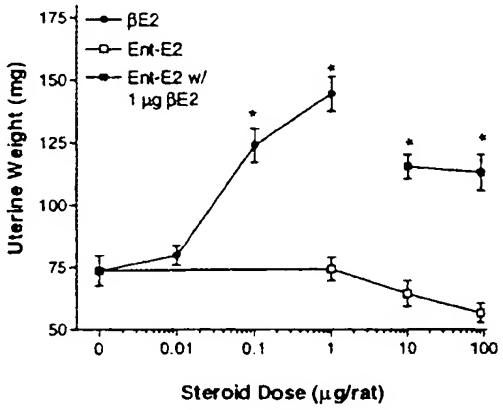

βE2
Ent-E2
Ent-E2 w/
1 μg βE2
Uterine Weight (mg)
175
150
125
100
75
50
0
0.01
0.1
1
10
100
Steroid Dose (μg/rat)

FIG. 16

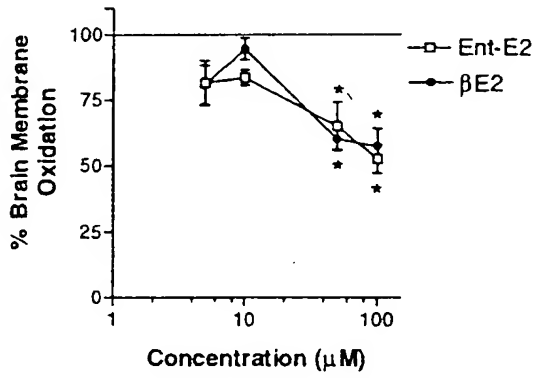

% Brain Membrane Oxidation
100
75
50
25
0
1
10
100
Concentration (μM)
Ent-E2
βE2

FIG. 17